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The efficacy of last-line and experimental antimicrobials against Stenotrophomonas maltophilia and the characterisation of resistant mutant

The efficacy of last-line and experimental antimicrobials against *Stenotrophomonas maltophilia* and the characterisation of resistant mutants

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Abstract

In recent years, *Stenotrophomonas maltophilia* has not only gained importance due to its increasing prevalence in human disease around the world but because of its intrinsic multidrug resistance, which makes this bacterium an interesting model to study mechanisms of drug resistance while informing rational drug design and changes to chemotherapy protocols.

Here, we aimed to identify novel mechanisms of resistance to ceftazidime, amikacin, levofloxacin, and minocycline. Overexpression of the chromosomally encoded serine-β-lactamase (SBL), L2, and metallo- β-lactamase (MBL), L1, is known to confer resistance to β-lactams, including ceftazidime. However, the mechanisms behind β-lactamase hyperproduction are not completely understood. Here, a new mechanism responsible for L1/L2 hyperproduction has been characterised: loss of function mutations in *mpl* which likely lead to accumulation of activator ligand for AmpR, the transcriptional regulator of L2 and L1. Additionally, for the first time a TonB energy-dependent mechanism is proposed for ceftazidime uptake where the alteration of a proline-rich region, deactivating TonB, is responsible for ceftazidime resistance in non-β-lactamase hyper-producers.

Although reduced antimicrobial permeability in *S. maltophilia* due to upregulation of efflux-pumps is well known, little is known about their regulation. Here evidence is provided to associate SmeYZ aminoglycoside efflux pump upregulation with ribosomal damage caused by mutations in the *rplA* gene, encoding a ribosomal subunit target of aminoglycosides and by ribosomal-acting agents such as gentamicin. Alterations in genes involved in lipid trafficking were found to be associated with SmeDEF efflux pump upregulation. Two novel ABC transporters were shown to be involved in levofloxacin resistance and reduced minocycline susceptibility; each is locally regulated by a two-component system. Minocycline was found to be the most promising therapeutic agent and resistant mutants or clinical isolates could not be found.

In addition to characterise last-line antimicrobials, the potential of non-classical β-lactamase inhibitors to restore β-lactam activity was studied. It was found that the diazabicyclooctane avibactam and the new bicyclic boronate **2** have potential to combat β-lactam resistance in *S. maltophilia* when compared to the traditional β-lactam based inhibitor, clavulanic acid based on microbiological, kinetic and structural evidence. In cases where the non-traditional inhibitors failed to restore β-lactam

antimicrobial activity (e.g. with ceftazidime) new combinations strategies (aztreonam/avibactam or aztreonam/bicyclic boronate **2**) or new inhibitors (MBL inhibitors in combination with meropenem) showed significant promise. In addition, the sideromimic modification of γ-lactam antibiotic, lactivicin (LTV-17) was found tp increase its antimicrobial activity 1000-fold against *S. maltophilia*. Although LTV-17 induces L1//L2 production, lactivicin is only slowly hydrolysed by β-lactamases. Therefore, LTV-17 antimicrobial activity is still strong against β-lactamase and efflux pump hyper-producing mutants, and extensively-drug resistant clinical isolates. Mutants with reduced LTV-17 susceptibility were identified, and were found to have the same loss of the TonB energy-transducer seen in ceftazidime resistant mutants. This work has given insight into the mechanisms of resistance in *S. maltophilia* to assist the optimisation and identification of possible candidates to combat this species in the clinic.

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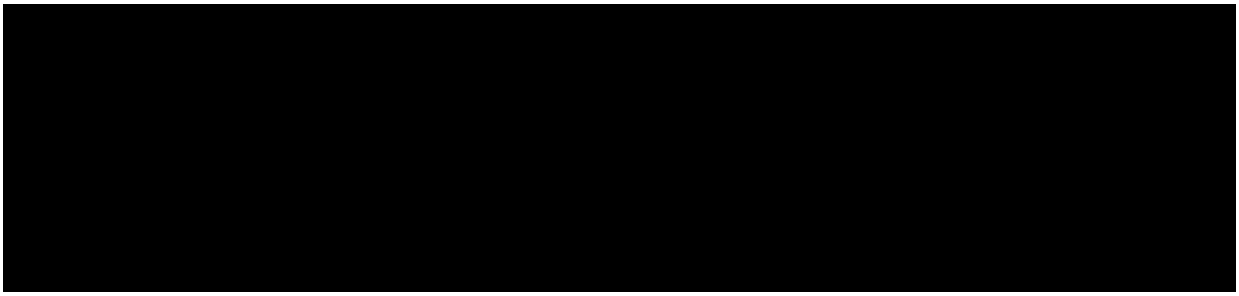
Last but not least, God and my loving and unconditional family that has always been with me regardless time and distance.

Dedication

To the memory of my grandparents and especially to the memory of my uncle Juan who instilled love and passion for biology in our family.

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University’s Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate’s own work. Work done in collaboration with, or with the assistance of, other, is indicated as such. Any views expressed in the dissertation are those of the author.



Publications and Presentations

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Calvopina K, Avison MB. 2018. Disruption of mpl Activates beta-Lactamase Production in *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* Clinical Isolates. Antimicrob Agents Chemother doi:10.1128/AAC.00638-18.

Hinchliffe P, Tanner CA, Krismanich AP, Labbe G, Goodfellow VJ, Marrone L, Desoky AY, **Calvopina K**, Whittle EE, Zeng F, Avison MB, Bols NC, Siemann S, Spencer J, Dmitrienko GI. 2018. Structural and Kinetic Studies of the Potent Inhibition of Metallo-beta-lactamases by 6-Phosphonomethylpyridine-2-carboxylates. Biochemistry **57:**1880-1892.

Calvopina K, Hinchliffe P, Brem J, Heesom KJ, Johnson S, Cain R, Lohans CT, Fishwick CWG, Schofield CJ, Spencer J, Avison MB**.** 2017. Structural/mechanistic insights into the efficacy of nonclassical beta-lactamase inhibitors against extensively drug resistant *Stenotrophomonas maltophilia* clinical isolates. Molecular Microbiology **106:**492-504.

Calvopina K, Umland KD, Rydzik AM, Hinchliffe P, Brem J, Spencer J, Schofield CJ, Avison MB**.** 2016. Sideromimic Modification of Lactivicin Dramatically Increases Potency against Extensively Drug-Resistant *Stenotrophomonas maltophilia* Clinical Isolates. Antimicrobial Agents and Chemotherapy **60:**4170-4175

Oral presentation

Role of *mpl* mutations in the beta-lactamase hyperesistant phenotype in *Stenotrophomonas maltophilia.* **Calvopina K,** Avison MB. (2017). 27th ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, Vienna-Austria. Presenting author.

Poster presentation

Proteomic-mediated detection of quinolone and aminoglycoside resistance mechanisms in *Stenotrophomonas maltophilia.* **Calvopina K,** Avison MB (2017). British Society for Antimicrobial Chemotherapy. Birmingham, UK

Proteomic-mediated detection of quinolone and aminoglycoside resistance mechanisms in *Stenotrophomonas maltophilia.* **Calvopina K,** Avison MB (2017). American Society for Microbiology. New Orleans, USA

Structural and mechanistic studies reveal effectiveness of non-classical β-lactamase inhibitors against extensively drug resistant *Stenotrophomonas maltophilia*. **Calvopina K,** Hinchliffe P, Brem J, Heesom KJ, Johnson S, Cain R, Lohans CT, Fishwick CWG, Schofield CJ, Spencer J, Avison MB. (2017). South West Structural Biology Consortium Meeting. Cardiff, UK

Structural and mechanistic studies reveal effectiveness of non-classical β-lactamase inhibitors against extensively drug resistant *Stenotrophomonas maltophilia*. **Calvopina K,** Hinchliffe P, Brem J, Heesom KJ, Johnson S, Cain R, Lohans CT, Fishwick CWG, Schofield CJ, Spencer J, Avison MB. (2017). 13th Beta-Lactamase meeting. L'Aquila, Italy

Structural Characterisation of L2 β-lactamase from *Stenotrophomonas maltophilia*. **Calvopina K,** Hinchliffe P, Brem J, Heesom KJ, Johnson S, Cain R, Lohans CT, Fishwick CWG, Schofield CJ, Spencer J, Avison MB. (2016). South West Structural Biology Consortium Meeting. Portsmouth, UK

Sideromimic modification of Lactivicin dramatically increases potency against extensively drug resistant *Stenotrophomonas maltophilia* clinical isolates. **Calvopina K,** Umland KD, Rydzik AM, Hinchliffe P, Brem J, Spencer J, Schofield CJ, Avison MB**.** (2016). Bristol Bridge. Bristol, UK. Presenting Author

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List of Abbreviations

CAS	Chrome Azurol S
CAZ	Ceftazidime
DIALS	Diamond light source
ESBL	Extended spectrum B-lactamase
IC50	Half maximal inhibitory concentration
IPTG	Isopropyl B-D-1-thiogalactopyranoside
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LGP	Ligand-gated porin
LTV	Lactivicin
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal inhibitory concentration
NB	Nutrient broth
Ni-NTA	Ni2+-nitriloacetic acid
PEG	Polyethylene glycol
RMSD	Root mean square deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOC	Super optimal broth with catabolite repression
TAE	Tris/acetate/EDTA
TCEP	Tris (2-carboxyethyl)
TEMED	Tetramethylethylenediamine
UDP	Uridine diphosphate
XDS	X-ray diffraction and spectroscopy
X-gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1 Introduction

1.1 Origin of antibiotics

Amongst the natural roles of secondary metabolites, the ecological role is only one explanation for their vast distribution (1). Secondary metabolites produced by microorganisms can be used to shape population density in a dynamic way. When secondary metabolites are antibiotics –natural products that eliminate other microorganisms- producers can prevent overgrowth of the community whilst non-secondary-metabolite producers may have mechanisms to avoid the antibiotic meaning that the mixed population reaches an equilibrium (2, 3). The human body also produces antimicrobial chemicals (part of the innate immune system) which are active against bacteria, viruses, archaea, and lower eukaryotic cells that humans may come into contact with (4, 5). Yet despite the existence of a potent innate immune system, humans are susceptible to microbes as a cause of disease. This might be through excessive growth of indigenous (also known as commensal) microbes which has been associated with cardiovascular diseases, cancer and even neurodegenerative diseases (6-8). However, the most notorious impact of microbes on human health throughout history, is the presence and/or the disproportional growth of exogenous microorganisms that are pathogenic, and so can cause specific diseases. Given that the adaptive immune system is frequently insufficient at dealing with this exogenous threat, it is unsurprising that human have turned to technology to regain some sort of control over pathogenic microorganisms. To do this, humans have, pursued antibiotics obtained from a natural environments and used them to 'tame' the microbial populations that live within them, and can attack them, and so doing they have extended human lifespan.

Alleviation of man's ailments empirically started in Nature, even before humans associated the detrimental effects of infections with microorganisms. The earliest applications of natural extracts are described in Mesopotamian records, which are still used today to treat coughs and parasitic infections (9). However, infection outbreaks, during history, quickly demonstrated that humans needed to be more proactive to tilt the balance. Although adoptions of public health measures like vaccination, quarantine, or pasteurization clearly reduced the rate of infections, it was observation of the antibiosis phenomenon that accelerated this process (10). Observation of antibioses gave rise to the term 'antibiotic' to dsecribe those substances that alter biochemical processes in bacteria and therefore inhibit cell growth (bacteriostatic) or

kill the bacterial cell (bactericidal) (11). Interest in the isolation of antibiotics increased and with it the scrutiny of natural sources, methods of chemical synthesis and more recently bioengineered systems.

Traditionally, natural compounds have constituted the platform for development of antibiotics. It is estimated that over the period 1981-2014, nearly 60% of the identified chemical entities with antimicrobial properties had a natural origin or were derived from a natural scaffold structures (12). It was Sir Alexander Fleming's and Selman Waksman's observations that pointed out fungi and bacteria as sources of antibiotics. In fact, these observations established the 'golden era' of antibiotic discovery (1940s-1960s) (**Figure 1.1**). The enormous success of soil-streptomycetes and soil-actinomycetes- antibiotics, against bacterial infections, quickly started to decline in the 1990s due to the rise and spread of antibiotic resistance (13). Nowadays, natural product discovery platforms are being enriched by exploring new environments, which in the past were not considered due to the limitations of recreating the growth conditions in the laboratory. Lichens, sponges, seaweed, corals and marine plants are providing interesting evidence for the next-generation of antibiotic scaffolds (14).

The first successful compound with antimicrobial activity in clinical use was chemically obtained. Paul Ehrlich whose interest in understanding how substances were being up-taken by organisms led to explore the antimicrobial activity of arsenicals. Ehrlich obtained a bioactive compound, Salvarsan that killed the causing agent of syphilis (*Treponema pallidum)*. Later, Salvarsan was known as the 'magic bullet' due to the killing effect it had on the target pathogen but not on the patient's healthy tissue. Following the same line of discovery, Prontosil was tested by Gerhard Domagk and Ernest Fourneau's groups and finally determined that the sulphonamide group in it was enough for the killing activity (15). Since then, research with synthetic antimicrobials has been carried out as an alternative way of generating innovative chemical skeletons after it became clear that searching for natural products led to continuous rediscovery of old molecules from natural platforms. Despite its innovation, the effectiveness of chemically synthesized antimicrobials has been limited due to the required chemical complexity of the molecules and with it restricted cell penetration properties. During the last 50 years only two successful classes of antibiotics have been chemically designed: fluoroquinolones and oxazilidinones (16).

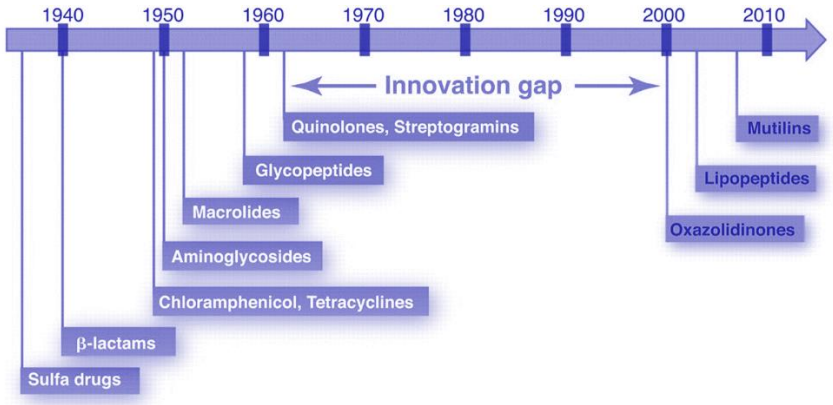


Figure 1.1 Timeline of Antibiotic Discovery

Describes the introduction of different classes of antibiotics. Adapted from (12)

Bioengineered systems have emerged as an approach to go beyond the limitations of natural and chemically synthesised antibiotics. In fact, combined efforts of synthetic biology and metabolic engineering have identified active biomolecules, with improved antimicrobial activity. The production of these secondary metabolites has been achieved due to the identification and manipulation of genetic elements that seem to be silent or cryptic, under specific conditions, and further tuning through manipulation of their respective regulatory elements in the biosynthetic pathways. The challenge remains to accurately identify these antibiotic biosynthetic genes (17).

The ancient, natural origin of antibiotics shows that microbes have been in contact with antibiotics for many millions if not billions of years. Certainly, exposure to antibiotics did not start after the introduction of antibiotics into clinical practice. However, this contact did increase during the modern antibiotic era. The close relationship that humans have developed with antibiotics, during the last seven decades, has led to dramatic benefits for human health. But also has led to the selection of new, and pre-evolved resistance to antibiotics in bacteria. Our relationship with antibiotics is not over, but it will experience changes based on lessons learnt from the past and the rise of antibiotic resistance in the future.

1.2 Use of antibiotics

Antibiotics have found an application in many different human activities and are not only limited to saving lives. However, antibiotics have an underpinning role in

modern medicine. Surgical site infections have reduced after establishing guidelines for the prophylactic administration of antibiotics. Likewise, the successful outcome of other medical treatments such as cancer chemotherapy has been partially due to the prevention or cure of infections in those patients whose immune systems are compromised by chemotherapy (18). Even in those geographical areas where modern medicine has not reached its peak and sanitation systems are still poor, administration of antibiotics has controlled the spread and reduced the severity of communicable infections (19). Evidently, the use of antibiotics has reduced morbidity and mortality but most important has been a pivotal factor to increase life expectancy when combined with the virtues of medicine.

The non-medical uses of antibiotics and synthetic antimicrobials include a wide range of activities from agriculture to domestic use. In animal husbandry, antibiotics have been used for growth promotion and as infection preventers where a large number of individuals are in contact. In plant agriculture, bacterial diseases are less prevalent; however, antibiotics are still used to protect the crops. In industries such as brewing production, the use of antibiotics increases starch fermentation by yeast by abolishing bacterial competition. In activities of daily living, numerous products that contain bioactive substances such as soap, shampoo, deodorants, facial cleaners, etc. kill potential harmful bacteria. The medley of anthropogenic activities represents a large fraction of the artificial presence of antibiotics in the ecosystem (20).

Understanding the success of antibiotics throughout history has also revealed, at a slow pace, valuable reasons to explain their failure. The usefulness humans have found in antibiotics, has triggered their overuse. Thus, the constant exposure of bacteria to antibiotics has only accelerated the evolution of the natural mechanisms of antibiotic resistance in bacteria. By 1946, Fleming had already foreseen this phenomenon when he stated: ‘There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react in some way acquiring fastness’.

1.3 Antibiotics and their targets

Understanding bactericidal effect of antibiotics is focused on the study of the drug-target interactions that inhibit cellular functions. According to the cellular target, current antibiotics can be classified into three different types. Bacterial cell envelope is disrupted by, for example β-lactams, glycopeptides and polymyxins. Antibiotics that inhibit protein synthesis include macrolides, chloramphenicol, aminoglycosides, and

tetracyclines. Synthesis of nucleic acids is inhibited by quinolones, rifampicin, sulphonamides and trimethoprim (21).

1.3.1 β-lactams

1.3.1.1 Mechanism of action

The most prescribed antibiotics in clinics are β-lactams. β-lactams represent 65% of the antibiotics market, comprising sales of US $15 billion per year (22, 23). Good activity against a wide range of Gram-positive and Gram-negative bacteria and good penetration in skin and soft tissues have made β-lactams a very popular choice in first-line therapy. β-lactams target, peptidoglycan (PG), a component present in the cell wall of Gram-positive and Gram-negative bacteria.

PG is made of repeating subunits of a disaccharide N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) connected by oligopeptide stems. The PG unit is transported to the periplasm and joins to the end of extending glycan chain by the action of transglycosylases. The stem oligopeptides bound to NAM are cross-linked to the neighbouring stem oligopeptides by transpeptidases. Penicillin-binding-proteins (PBPs) are responsible for the polymerization and cross-linking of the peptidoglycan chains. As well as being transglycosylases, PBPs can have a DD-peptidase activity that includes DD-transpeptidase, DD-carboxypeptidase or DD-endo-peptidase activity. Some PBPs have dual activity. During transpeptidation; the double lysine-serine system, present in the active site of high-molecular weight PBPs such as PBP-1, PBP-2, PBP-3, is responsible for acylation and deacylation. Here, the penultimate D-Ala residue from the stem pentapeptide acylates the PBP obtaining an acyl-enzyme complex which leads to deacylation and removal of the terminal D-Ala residue, resulting in cross-linking of the reactive acyl-enzyme complex with the amino group of the third residue of the neighbouring stem oligopeptides. In Gram-negative bacteria the cross-linking is direct while in Gram-positives, such as *Staphylococcus aureus,* it occurs through a pentaglycine bridge or in the case of *Enterobacterium faecium* via a single D-Asp (24-27).

β-lactams exert their bactericidal action on the transpeptidase PBPs anchored to the outer surface of the cytoplasmic membrane after entering into the cell via hydrophilic channels (porins) present in the outer membrane (**Figure 1.2**). The chemical structure of β-lactams mimics the D-alanyl-D-alanine terminus of the stem peptide that reacts with DD-peptidase PBPs (28). The main difference relates to the covalent binding of the carbonyl group, present in the β-lactam ring, with the serine

residue of the active site of PBPs which results in a stable acyl-enzyme complex which is no longer reactive. On its own, inhibition of cross-linking does not abolish cell wall biosynthesis but added to the active state of cell wall lytic transglycosylases, integrity of the cell wall is reduced and ultimately leads to cell death.

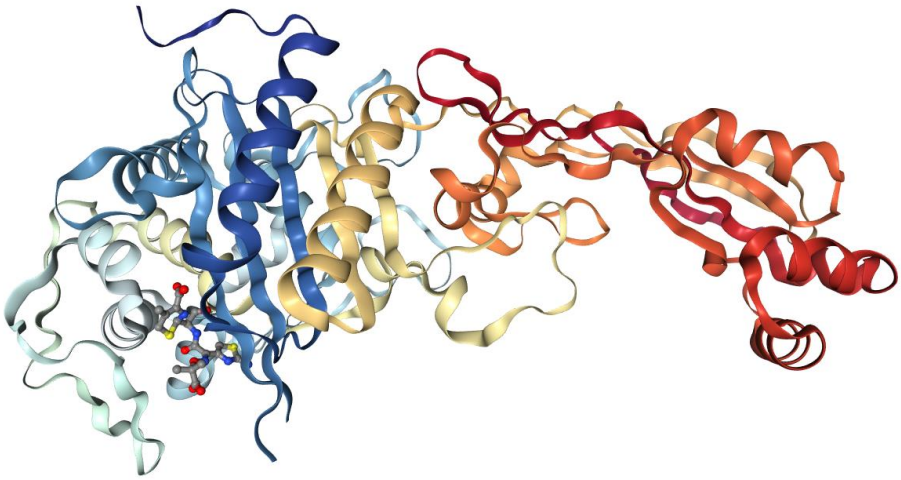


Figure 1.2 PBP-3 complexed with ceftazidime

Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

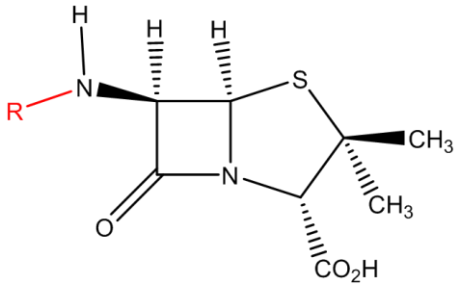
1.3.1.2 Classification

Early work to obtain industrial quantities of penicillin showed that different producer strains grown at certain conditions produced different penicillin derivatives. Howard Florey's group obtain 2-pentenylpenicillin from an English strain (*Penicillium notatum)* while in the United States, benzyl-penicillin was obtained from a different strain (*Penicillium chrysogenum*). However, the core structure in both types of penicillin was the β-lactam ring which results from the condensation of three amino acids (L-α-aminoapidic acid, L-cysteine and L-valine) (28, 29). Based on these findings, chemists were interested on structure modifications to improve absorption in the human body but most important to increase the spectrum of antimicrobial activity (12, 30).

1.3.1.3 Penicillins

Penicillins contain a bicyclic 'penam' nucleus (**Figure 1.3**). This core results from the phusion of the rings of a β-lactam and a thiazolidine, and an acyl side-chain. In nature they are produced by some *Penicillium* and *Aspergillus* species (29).

However, fermentation studies by workers at Beecham Pharmaceuticals in 1958-1960 demonstrated that chemical synthesis of penicillin was not only possible but could also improve its antimicrobial activity (30, 31). A penicillin intermediate, 6-amino-penicillanic acid (6-APA), was obtained through fermentation from where different penicillin analogues could be obtained by its acylation with acid chlorides. The reasoning behind addition of different acyl side-chains to 6-APA was preventing β-lactam ring opening. The carbonyl group present in the ring can react with a neighbouring acyl group which ultimately renders an opened β-lactam ring (32). The strategy to prevent the opening is to add a bulky acyl side chain which gave place to the following penicillin derivatives: penicillinase-sensitive, penicillinase-resistant, aminopenicillins, antipseudomonal penicillins, and extended spectrum penicillins (33).



6-APA

Figure 1.3 Penicillin core 6-amino-penicillanic acid

Penicillin G (benzylpenicillin) and penicillin V (phenoxymethyl-penicillin) belong to the penicillinase-sensitive group (**Figure 1.4**). β-lactamases, formerly known as penicillinases, are enzymes that can open the β-lactam ring which leads to its inactivation. The first penicillinase-resistant penicillin was methicillin which was obtained by the addition of a bulky acyl-side chain. Additional chemical modifications gave place to oxacillin, cloxacillin, dicloxacillin which offered a gamut of pharmacokinetic properties but with a narrow spectrum of activity. Aminopenicillins such as amoxicillin and ampicillin are not only effective against Gram-positive bacteria but also Gram-negatives, nevertheless they are still sensitive to β-lactamases. Carbenicillin and ticarcillin are resistant to some β-lactamases and possess a wider spectrum of activity against Gram-negative pathogens including *Pseudomonas aeruginosa*. The extended spectrum penicillins are not only effective against 95% of *P. aeruginosa* isolates but also Enterobacteriaceae especially *Klebsiella* spp. isolates (32).

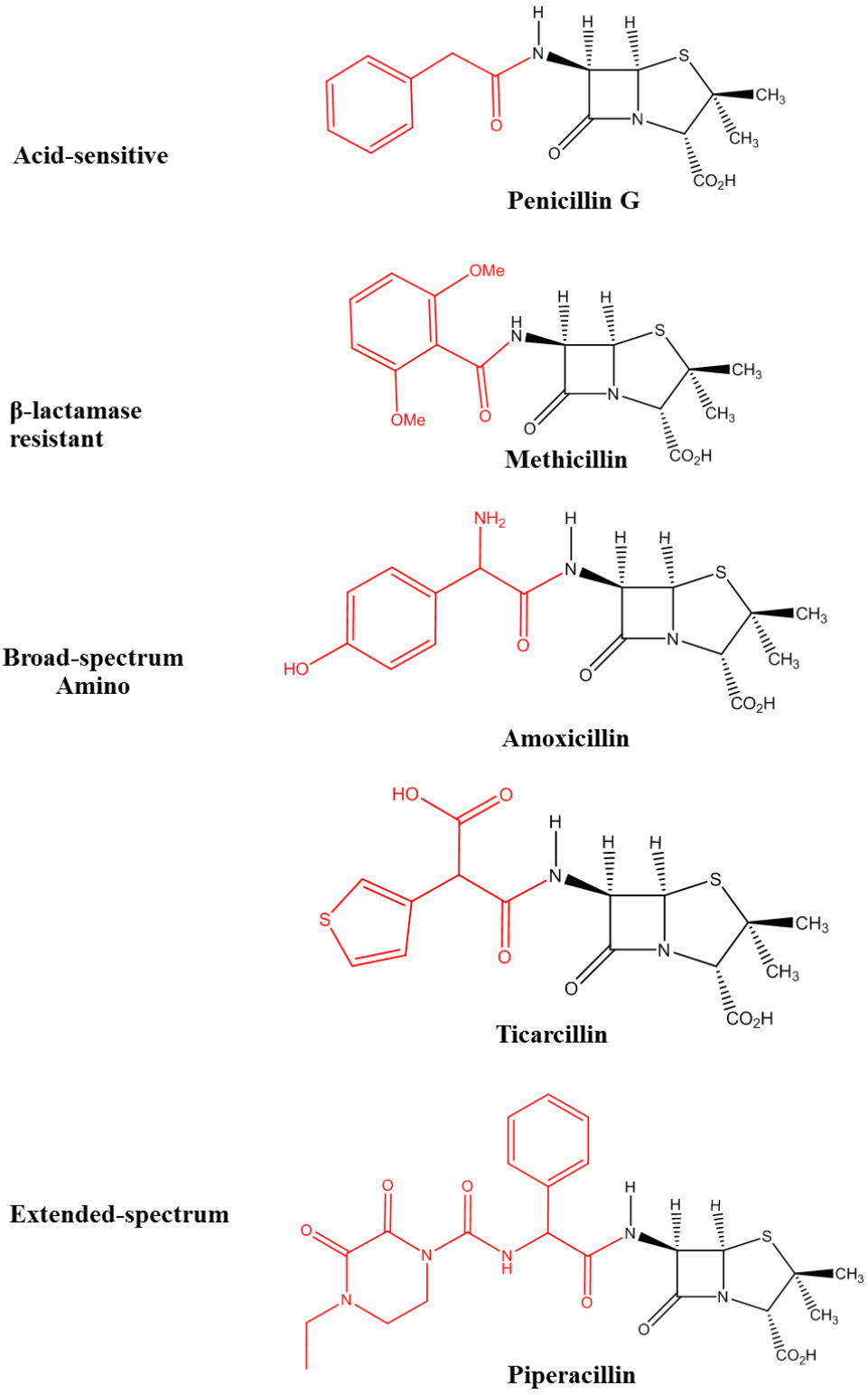
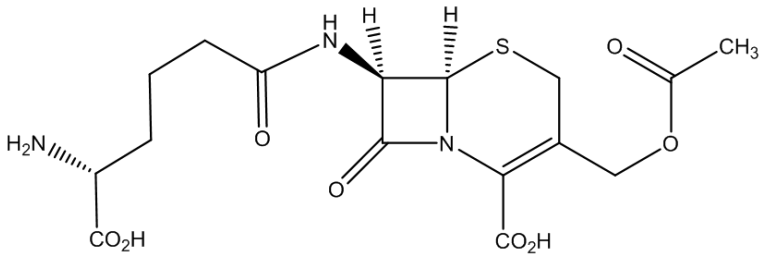


Figure 1.4 Representatives of penicillin groups

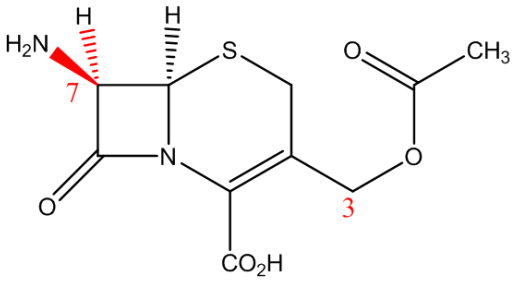
Modification are shown in red and the penicillin core in black

1.3.1.4 Cephalosporins

Cephalosporin C was recovered from a fungus (C*ephalosporin acremonium*) found in sewer waters on the Sardininan Island in 1948. Different from penicillins, the structure of cephalosporins include a six-membered dihydrothiazine ring, instead of a five-membered ring, fused to the β-lactam ring. Synthetic tailoring, to obtain cephalosporin analogues, was not an easy task since 7-ACA (7-amino-cephalosporin acid) could not be obtained by fermentation. Chemical modifications in 7-ACA were only possible, when 7-ACA was obtained in enough quantities as a result of a new method of chemical hydrolysis to eliminate the side chain of Cephalosporin C. Such modifications can occur in three different places: the carbon atom 7 or the acyl side chain of the β-lactam ring or the acetoxymethyl chain of the dihydrothiazine ring (carbon atom 3) (**Figure 1.5**) (32).



Cephalosporin C



7-ACA

Figure 1.5 Cephalosporins core 7-aminocephalosporin

Cephalosporin C is the original cephalosporin that rendered 7-ACA to allow newer generations of cephalosporins.

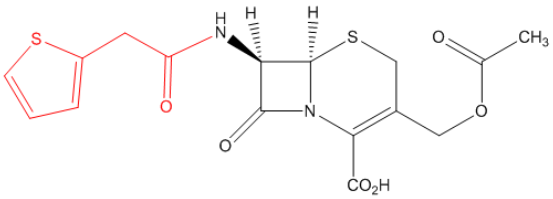
First-generation cephalosporins include cephalothin (2-thiopheneacetyl derivative), the first cephalosporin analogue, which was the result of a variation of the 7-acylamino acid chain. In general, cephalothin has low affinity to PBPs of Gram-negative bacteria and it is only active against some Gram-positive and Gram-negative

bacilli but it is resistant to β-lactamases produced by *Staphylococcus aureus*. Cephaloridine instead, emerged from the substitution of the acetoxymethyl chain with a pyridinium group that increases activity against Gram-positive organisms. The methyl substitution at the same position with the appropriate 7-acylamino group gave place to cephalexin to increase the gut wall uptake but maintaining its antimicrobial activity (34, 35).

Second-generation cephalosporins include Cephamycin C which was the first β-lactam obtained from a bacterium (*Streptomyces clavuligens*). A methoxy variation at the carbon atom 7 with a variation of the side chain gave place to cefoxitin. Cefoxitin has an increased potency against Gram-positive and Gram-negative bacteria and reduced susceptibility to the hydrolytic activity of β-lactamases and mammalian esterases. Although the introduction of the α-oxymino group in the 7-acyl chain increases the affinity for PBP-3, e.g. to make cefuroxime; the antipseudomonal activity is still low (34, 35).

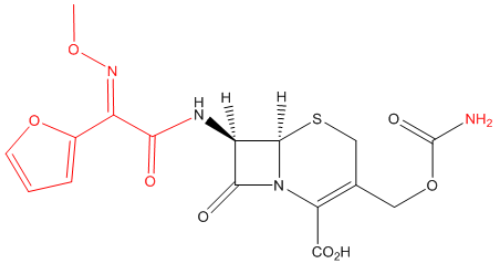
Third-generation cephalosporins such as ceftazidime incorporated a new thiazolidine ring in the 7-acyl chain, which improved its bacterial penetration without compromising its target affinity. In Gram-negative organisms, low-dose ceftazidime leads to bacterial filamentation formation due to PBP-3 inhibition whereas at high concentrations inhibits PBP-1 and causes quick bacteriolysis (15, 36). The appearance of β-lactamases capable of hydrolysing third-generation cephalosporins prompted a fourth generation of cephalosporins by maintaining the zwitterionic structure of the third-generation cephalosporins but adding an amino-thiazole side chain. Cefepime or BMY-28142 was more stable than other broad-spectrum cephalosporins against β-lactamases and had antipseudomonal activity (37, 38). Currently, use of fifth-generation cephalosporins such as ceftobiprole, is only approved in Europe. Ceftobiprole includes a vynilpyrrolidinone at carbon 3, which augments affinity for PBP-2 and explains its distinguished efficacy against methicillin-resistant *S. aureus* strains (**Figure 1.6**) (37, 39)

I Generation



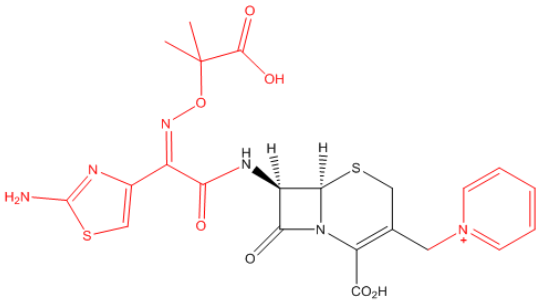
Cephalothin

II Generation



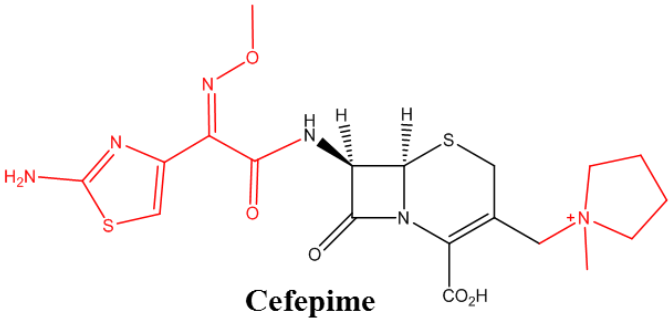
Cefuroxime

III Generation



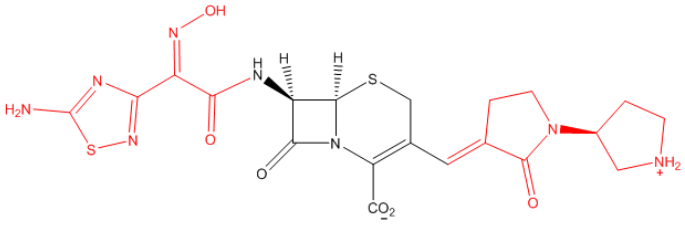
Ceftazidime

IV Generation



Cefepime

V Generation

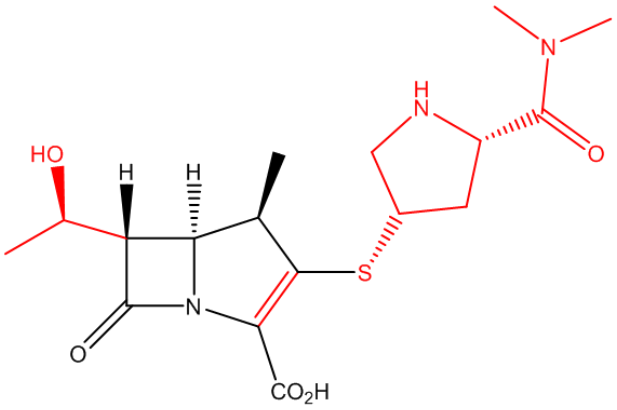


Ceftoprobiole

Figure 1.6 Representatives of cephalosporin groups

1.3.1.5 Carbapenemens

Carbapenems include a β-lactam ring fused to a five-membered ring (pyrroline) but they differ from the penicillin and cephalosporin in three ways. In the five-membered ring, presence of a double bond between C2 and C3 and substitution of the traditional sulphur atom (in penicillins and cephalosporins) with a carbon atom, enhance the antimicrobial activity. Additionally to these changes, a hydroxyethyl group replaces the 7-acyl side chain, which is also responsible for the activity against multiple PBPs of Gram-Positive and Gram-negative bacteria, including *P. aeruginosa* and its slow hydrolysis in presence of β-lactamases (40). Thienamycin was the first carbapenem obtained from *Streptomyces cattleya* but it was chemically unstable. The first carbapenem analogue that did not have this instability was imipenem (41) which was obtained after the addition of a formamidine group at position C3. In general, imipenem has higher affinity for PBP-1a and works better against Gram-positive bacteria. Imipenem intravenous administration was successful therapeutically only when combined with the dehydropeptidase inhibitor cilastin, because imipenem is inactivated by renal dehydropeptidase DPH-1 (42), and cilastin inhibits this enzyme. Meropenem does not need to be administered with cilastin due to the presence of a methyl group in the pyrroline ring. The modification of the C2 side chain that includes a pyrrolidine group confers broad-spectrum activity, which is relative higher against Gram-negative pathogens when compared to imipenem (**Figure 1.7**) (43, 44). Doripenem does not require co-administration since it shares structural similarities with meropenem. When compared to imipenem, doripenem has a higher antipseudomonal activity due to its affinity to PBP-2 and PBP-3 in this particular species (45). Ertapenem instead has limited activity against *P. aeruginosa* but still effective against Gram-positive aerobic bacteria and Gram-negative enterobacteria producing β-lactamase. Ertapenem only requires administration once a day and it is used as a complement to imipenem and meropenem (43).



Meropenem

Figure 1.7 Representative of carbapenems

1.3.1.6 Monobactams

Monobactams were discovered because of a screening that included soil bacteria such as: *Acinetobacter, Agrobacterium, Chromobacterium, Pseudomonas. Chromobacterium violaceum* was responsible of the production of the simplest form of monobactam 3-AMA (3-Aminomonobactamic acid) (**Figure 1.8**). Opposite to the previous structures of penicillins and cephalosporins, monobactams only have the β-lactam ring. Despite the weak activity, monobactams showed to have affinity for PBPs and surprisingly great stability in presence of β-lactamases. Aztreonam is the only monobactam commercially available and it is synthetic. The sulphur atom added to the β-lactam ring is responsible of its increased activity whilst the carbonyl and methyl groups in C4 confers resistance to β-lactamase activity (**Figure 1.8**). Finally, the acyl side chain improves activity against Gram-negative pathogens (46-49).

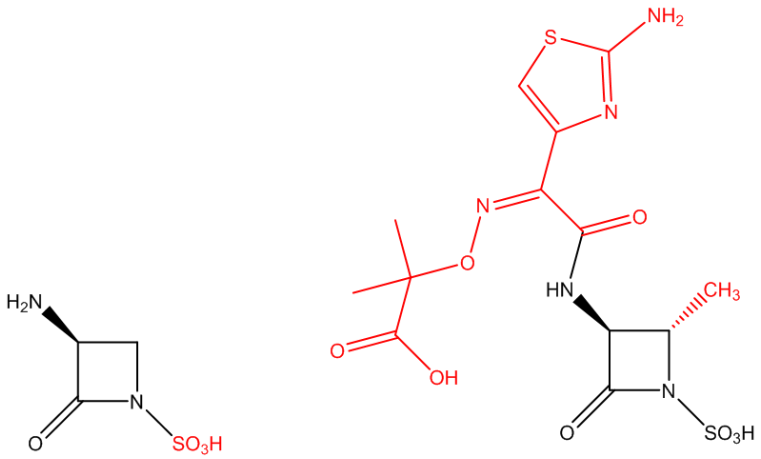


Figure 1.8 Core and representative of monobactams

1.3.2 Quinolones

1.3.2.1 Mechanism of action

Over the past few decades, quinolones rose to become one of the most prescribed classes of antibacterial drugs after β-lactams (23, 50). Quinolones are based on the antimicrobial activity of a derivative from quinine production, which was initially considered a waste product (51). Further optimisation, lead to the first useful analogue, nalixidic acid, which prompted the synthesis of approximately 10000 quinolones that over history have yielded more than 25 clinically approved compounds with broad spectrum activity (15). Quinolones target bacterial topoisomerases, responsible of modulating the separation of the DNA strands during replication and transcription (52).

In *E. coli,* hydrophilic quinolones are up-taken through the porin OmpF (53) and have their action by the inhibition of topoisomerase II, also known as DNA gyrase; and topoisomerase IV (**Figure 1.9**). In an ATP-dependent process, DNA gyrase is responsible of generating negative supercoils in DNA. Negative supercoils, opposite to the direction of the DNA twist (54), alleviate the generated tension of the positive supercoils in the regions ahead and behind the replication fork during DNA synthesis (52, 55). DNA gyrase is a heterotetramer enzyme, which comprises two identical subunits (two GyrA and two GyrB). GyrA breaks the DNA to form a transitory opening through which the transported DNA segment can pass and supercoil. GyrA

is also responsible of re-sealing the 4-base pair gate whilst GyrB presents an ATPase activity. Although topoisomerase IV is also responsible for relaxing positive supercoils generated during DNA synthesis, its primary role is to unlink the resulting new DNA molecules at the end of replication. Topoisomerase IV is also a heterotetramer formed by ParC and ParE subunits. DNA gyrase and Topoisomerase IV form a complex with the DNA strands, between the tyrosine residues present in the active site and the 5' end of the DNA break. Quinolones interact with the DNA-gyrase or DNA-topoisomerase IV complexes due to the presence of an Mg^{2+} ion. The water-Mg^{2+} ion bridges allow the interaction of quinolones and the serine and acidic residues of the complex. The quinolone-binding to GyrA changes the conformation of the enzyme to prevent the re-ligation of the broken DNA strands generated during the creation of the transient gate (52, 56), but ATP hydrolysis is not affected. In contrast, other topoisomerase-inhibitors such as the naturally occurring coumarins (novobiocin and coumermycin) bind to GyrB to interfer with supercoiling by preventing ATP use (57).

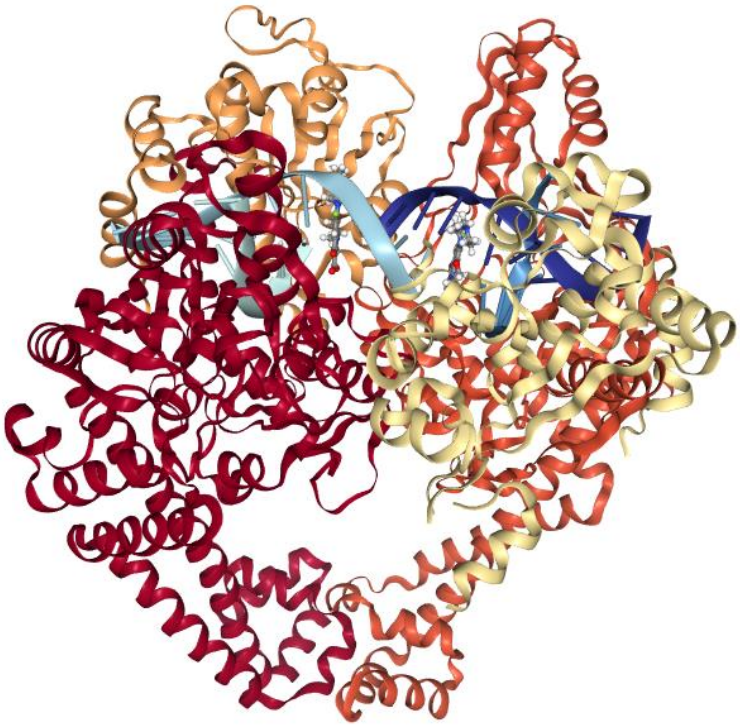


Figure 1.9 Topoisomerase IV-DNA –levofloxacin complex (PDB 3RAE)

Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

1.3.2.2 Classification

The quinolone group encompasses four generations of analogues. The first generation includes a naphthyridone, nalidixic acid, which presents a fused γ-pyridone and benzene rings(58). Different from the original quinolone, isolated from antimalarial-drug production, naphthyridones possess a nitrogen atom in the benzene ring instead of the carbon atom C8 (51) (**Figure 1.10**). Initially, nalidixic acid had a limited use due to its narrow activity spectrum. Later, nalidixic acid became popular to treat urinary tract infections caused by enterobacteria, especially in cases of bacterial resistance to other antibiotics used at the time (59). However, rapid emergence of resistance to nalidixic acid urged the synthesis of analogues (60). Both classes, naphthyridone and quinolones, were improved by the addition of a fluorine atom to the C6 position of the core structure. Improvement was evident by increased DNA gyrase inhibition and antimicrobial potency (51). However, quinolone classification tends to be restricted to fluoroquinolones since fluorinated naphthyridones are not common in developed countries (61, 62). The first fluoroquinolone obtained was flumequine. Flumequine preserved the good activity nalidixic acid had against Gram-negative but offered the first approach to improve activity against Gram-positive bacteria (60). The second generation of fluoroquinolones includes changes at position C7 and N1(51). Piperazine at position C7 in norfloxacin improved antimicrobial activity against Gram-negative due to the direct interaction with topoisomerase II and IV(63). Norfloxacin was considered the first broad-spectrum fluoroquinolone. Nevertheless, its poor tissue penetration limited its use to genitourinary diseases(64). The addition of a cyclopropyl group to N1 position gave place to ciprofloxacin, which demonstrated to have an extended spectrum of activity, including anti-pseudomonal activity, better tissue penetration and thus improved activity outside the urinary tract (51, 64). Ofloxacin contains a methyl group in an oxazine ring that links N1 and C8. Interestingly, the *S* isomer has two-fold increased activity that the *R* isomer(53). The *S*-isomer of ofloxacin, levofloxacin, was assigned to the third-generation group since its activity against Gram-positive and Gram-negative bacteria was higher which also implied the administration of a unique dosis per day (53, 62). Fourth-generation fluoroquinolones are mainly prescribed for respiratory infections since they present improved activity against Gram-positive bacteria and anaerobes (62). Moxifloxacin contains an azabicyclo at C7, a metoxy group at C8 and a cyclopropyl group at N1 (51) (**Figure 1.11**).

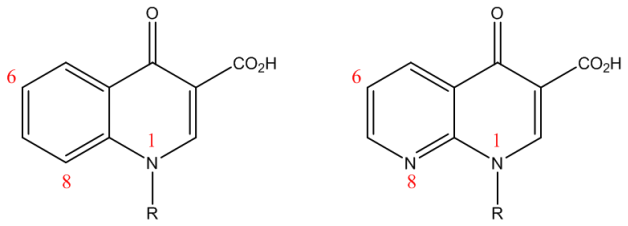
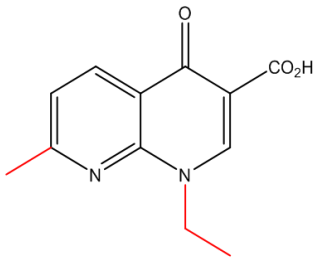


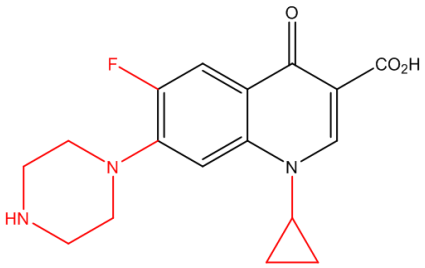
Figure 1.10 Quinolones core

I Generation



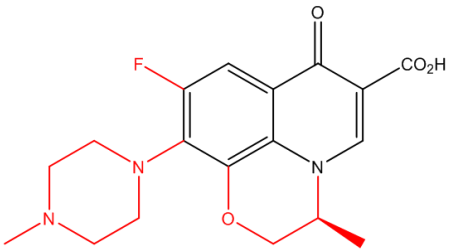
Nalidixic acid

II Generation



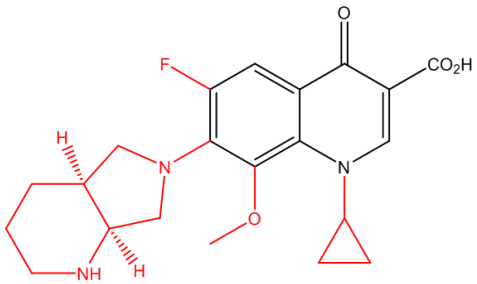
Ciprofloxacin

III Generation



Levofloxacin

IV Generation



Moxifloxacin

Figure 1.11 Representatives of quinolones

1.3.3 Tetracyclines

1.3.3.1 Mechanism of action

Tetracycline use around the world is difficult to determine. In developing countries, consumption of tetracycline increases due to its low cost (23, 65). However, the underestimation of its use as a growth promoter for swine, poultry and in aquaculture alters the final values. This is particularly certain for countries like the United States, China and India, but not Europe where growth promoting uses of antibiotics are banned (66). In fact, in some European countries the general trend of antibiotic consumption has decreased such the case of Norway, Sweden and Finland (67). In 1947, the first member of the tetracycline group, chlortetracycline or aureomycin, was isolated from *Streptomyces aureofaciens* (68) whilst oxytetraycline or terramycin was obtained from *Streptomyces rimous*. Soon, these tetracyclines became very popular due to their broad-spectrum bacteriostatic effect on Gram-positive, Gram-negative, anaerobes, and intracellular pathogens. Whilst the typical mode of action of tetracyclines prevents elongation of the peptide chain by binding to the 30S ribosomal subunit (32, 69), the atypical binding involves interaction with the bacterial cytoplasmic membrane (70).

Tetracycline uptake in Gram-negative bacteria depends on porins and its lipophilic chemical structure, which allows its passage through the inner membrane of Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, positively charged tetracycline-Mg^{2+} complexes traverse OmpF and OmpC porin channels and accumulate in the periplasm. Once in the periplasm the coordination complex releases the uncharged and lipophilic tetracycline, which crosses the cytoplasmic membrane by action of the proton motive force. Once in the cytoplasm, tetracycline is chelated by Mg^{2+} and the resulting complex is more likely to bind to the ribosome (71). Tetracycline binds to the S7 protein and the 16S rRNA, in the aminoacyl site (A-site) of the 30S ribosome (**Figure 1.12**). Here, the tetracycline binding prevents the association of the aminoacyl tRNA to the A-site of the ribosome. Tetracycline binding also inhibits the binding of release factors 1 and 2, which are responsible of the liberation of the resulting peptide chain (71, 72)

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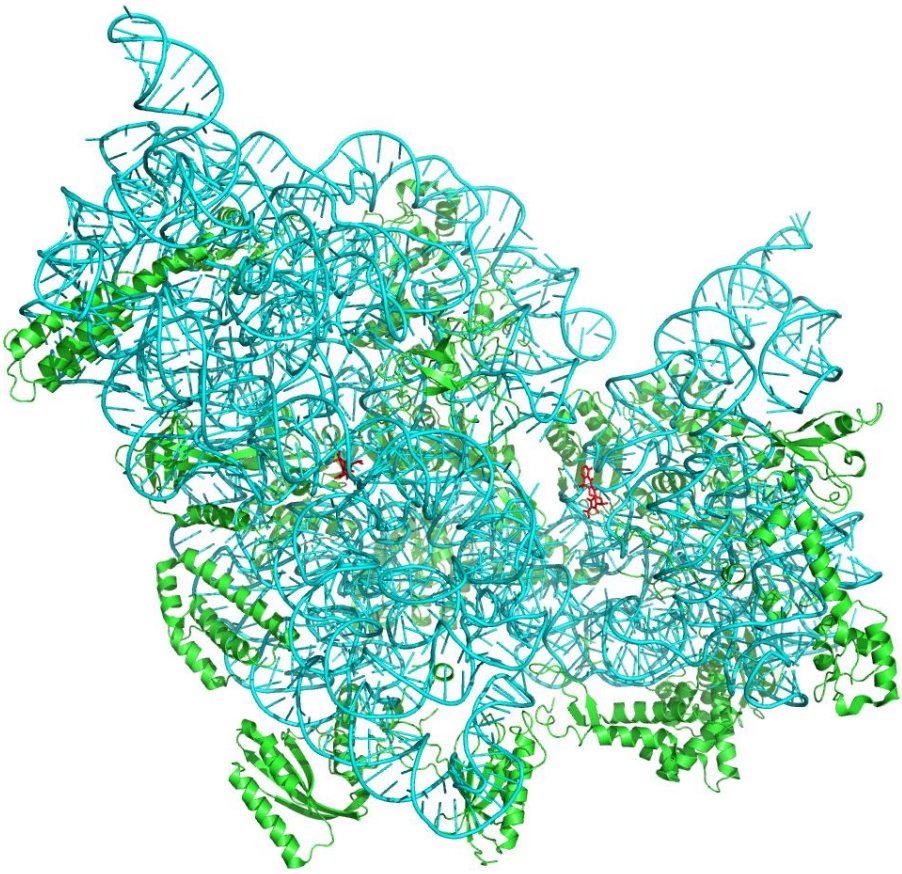
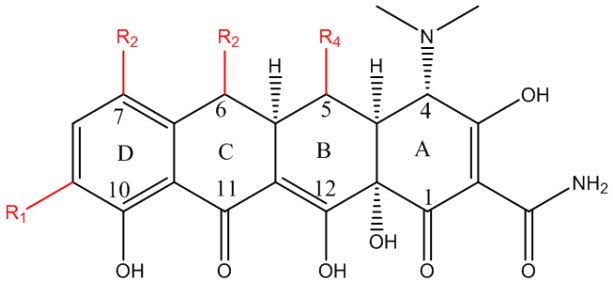


Figure 1.12 30S ribosome complexed with tetracycline (PDB 1HNW)

Molecular visualization with PyMOL. Tetracycline is highlighted in red

1.3.3.2 Classification

Tetracycline structure presents a basic core formed by naphthacene, four linearly fused aromatic rings (A, B, C, and D), where other functional groups have been attached to improve tetracycline antimicrobial activity (71). The minimal pharmacore is 6-deoxy-6-demethyltetracycline from where all generations are derived (**Figure 1.13**).

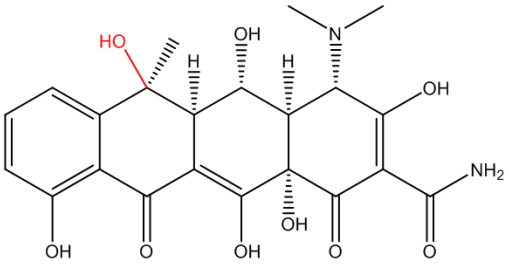


6-deoxy-6-demethyltetracycline

Figure 1.13 Tetracyclines core

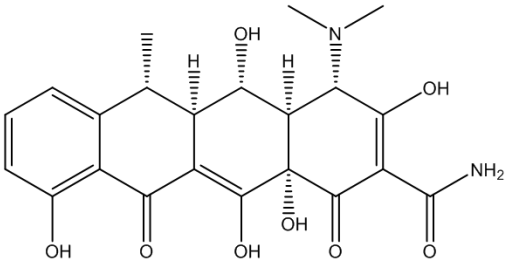
The first generation of tetracyclines include chlortetracycline, oxytetracycline, and tetracycline. Chlortetracycline lacks a hydroxyl moiety at C5 in ring B but has a chlorine atom at C7 in ring D when compared to oxytetracycline. The simplest structure of the group is a chlortetracycline derivative that has lost its chlorine atom, tetracycline. In fact, chemical synthesis of tetracycline and posterior identification as a precursor of chlortetracycline during fermentation process confirm improved antimicrobial activity when compared to other members of the group. Second-generation tetracyclines include semi-synthetic tetracylines that emerged as an alternative to reduce toxicity and improve antimicrobial activity. Modification of the C-ring in oxytetracycline render methacycline, which was later used to obtained doxycycline. Other attempts to potentiate tetracycline involved manipulation of bioengineered strains from where demeclocycline was obtained and later used to produce a different intermediate, sancycline. Further modifications in C7, such as the addition of a dimethylamino group gave place to minocycline. Third-generation tetracycline includes complete synthetic analogues such as tigecycline. Tigecycline is minocycline derivative with a glycylamide moiety at C9 (69, 73) (**Figure 1.14**).

I Generation



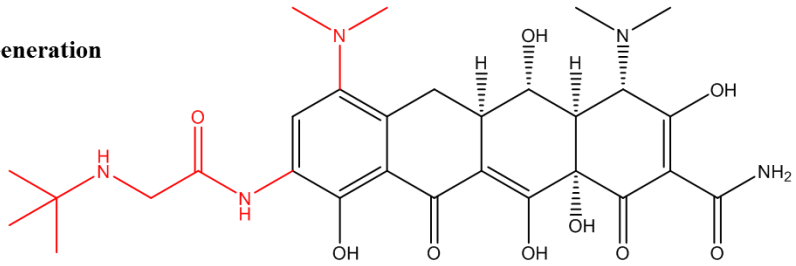
Oxytetracycline

II Generation



Doxicycline

III Generation



Tigecycline

Figure 1.14 Representatives of tetracyclines

1.3.4 Aminoglycosides

1.3.4.1 Mechanism of action

In 2014, the size of the global market for aminoglycosides was estimated to be USD 1.1 billion and it is expected to increase to USD 1.68 billion by 2022. Aminoglycosides are broad-spectrum antibiotics whose usage is likely to raise due to veterinary applications and administration for tuberculosis eradication (74). In 1944, streptomycin was the first aminoglycoside isolated obtained from *Streptomyces griseus*. Soon, streptomycin became the most important antibiotic after penicillin due to its effectiveness against tuberculosis (75). Aminoglycosides are polycationic

pseudo-oligosaccharides that alter protein synthesis by binding to the 30S ribosome (76).

Aminoglycosides cannot cross the outer membrane through porins due to its size. Instead, aminoglycosides rely on its displacing action on Mg^{2+} bridges, between lipopolysaccharides, to traverse the outer membrane. Penetration through the cytoplasmic membrane implies an energy-dependent phase, EDP-I (77). Once in the cytosol, EDP-II allows aminoglycosides to bind to the conserved A-site present in 16S rRNA present in the small subunit 30S (**Figure 1.15**). Whilst the formation of the translation initiation complex remains intact, the proofreading process of the peptide chain changes. The aminoglycoside binding modifies the conformation of an internal loop in the A-site, which allows the integration of unrelated tRNA. Thus, aminoglycoside binding contributes to codon misread and inhibition of the translocation of the tRNA-mRNA complex from the A-site to the P-site, which alters the elongation of the peptide chain (77, 78). Later, these erratic proteins can be integrated into the cytoplasmic membrane, which only increases the passage of more aminoglycoside (78).

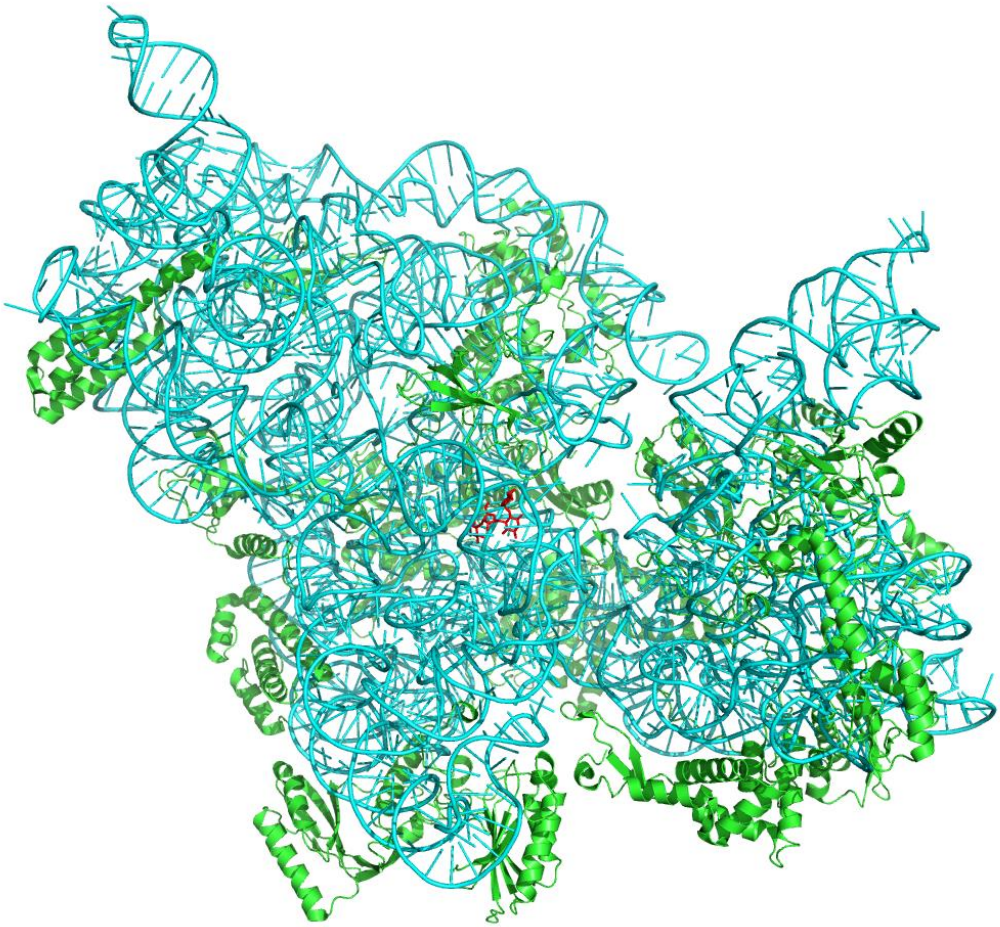
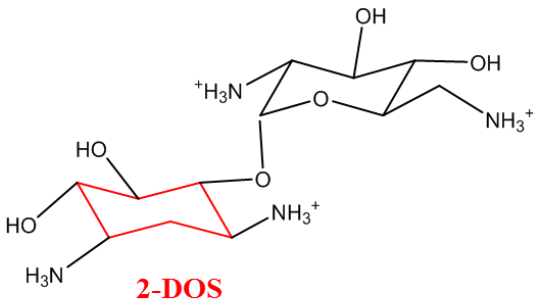


Figure 1.15 30S complexed with paranomycin (1FJG)

Molecular visualization with PyMOL. Paranomycin is highlighted in red

1.3.4.2 Classification

Natural and semi-synthetic aminoglycosides have a chemical core, which is neamine. Neamine is formed by a six-member aminocyclitol ring and a glucosaminopyranose (79). The aminocyclitol ring contains 2-deoxystreptamine (2-DOS), 1, 3 diamino groups and three or four hydroxyl groups to provide the link to aminosugars (**Figure 1.16**). In fact, the stereochemistry of the glycosidic bond between the neamine core and the carbohydrate is crucial for antibacterial activity of aminoglycosides (80).

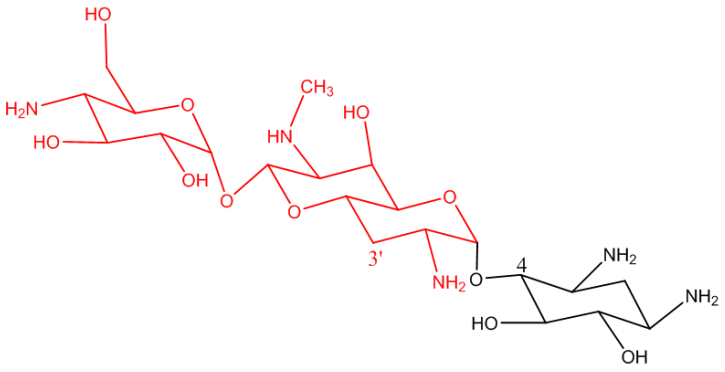


Neamine

Figure 1.16 Aminoglycosides core

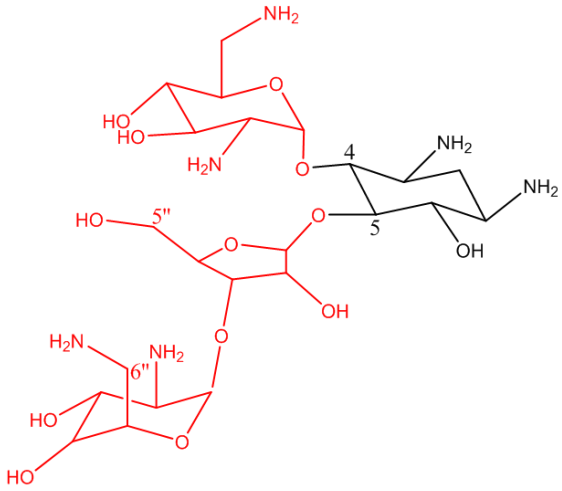
According to the chemical modification on the neamine core, aminoglycosides can be grouped in three different classes: 4-monosubstituted, 4,5-disubstituted, and 4,6-disubstituted (81). Apramycin represents monosubstituted aminoglycosides. In particular, apramycin has a veterinary application and it is the only aminoglycoside with a bicyclic ring linked to the neamine core. Neomicyn represents the group of 4, 5-disubstituted aminoglycosides, but the group contains three subclasses according to the chemistry of their third ring additional modifications. Neomycin was originally isolated from *Streptomyces fradiae.* Administration of neomycin is not intravenous due to nephrotoxicity and ototoxicity. Kanamycin and tobramycin belong to one of the two subclasses of 4, 6-disubstituted aminoglycosides whilst gentamicin and amikacin belong to the other. Kanamycin was isolated from *Streptomyces kanamyceticus,* gentamicin from *Micronospora purpurea*, tobramycin from *Streptomyces tenebrarius* and amikacin is a semi-synthetic aminoglycosides derived from kanamycin. The difference between both groups responds to the chemistry of the third ring and the additional modifications. The third group of aminoglycosides is widely used in combination to treat infections caused by Gram-negative, Gram-positive and non-typical mycobacterial infections including *P. aeruginosa, Salmonella* and *Enterobacter* species (82, 83) (**Figure 1.17**).

4-substituted



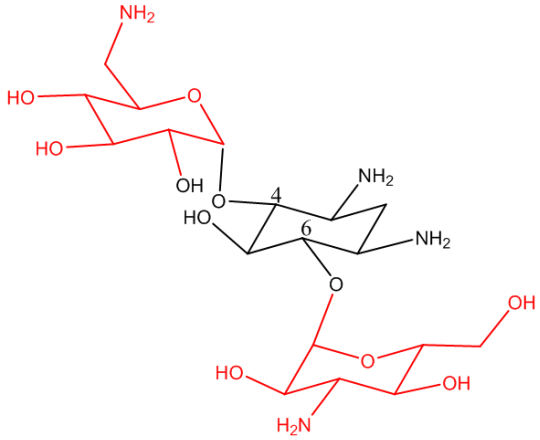
Apramycin

4,5-substituted



Neomycin

4,6-substituted



Kanamycin A

Figure 1.17 Representatives of aminoglycosides

1.4 Antibiotic Resistance

As previously discussed, because antibiotics are or are derived from natural products, many bacteria have been exposed to antibiotics for millennia. This means that antibiotic resistance is also ancient. For example analysis of bacteria in ancient permafrosts has revealed collections of antibiotic resistance genes, which date back millennia (84). This ancient origin of antibiotic resistance means that it has been with us since the first use of these drugs. At the end of the 1930s, sulfonamide resistance was reported in clinical practice as soon as it was introduced, the same mechanism that dominates 80 years later. In the 1940s, it was acknowledged that overuse of penicillin G promoted the selection of penicillinase producer strains. By the 1950s, common pathogens such as *S. aureus* had acquired resistance to all the available antibiotics of that time. A decade later, the introduction of semisynthetic versions of the previous failed antibiotics gave some hope. Shortly after introduction of new generations of antibiotics, new and improved mechanisms of resistance were reported (85) (**Figure 1.18**).

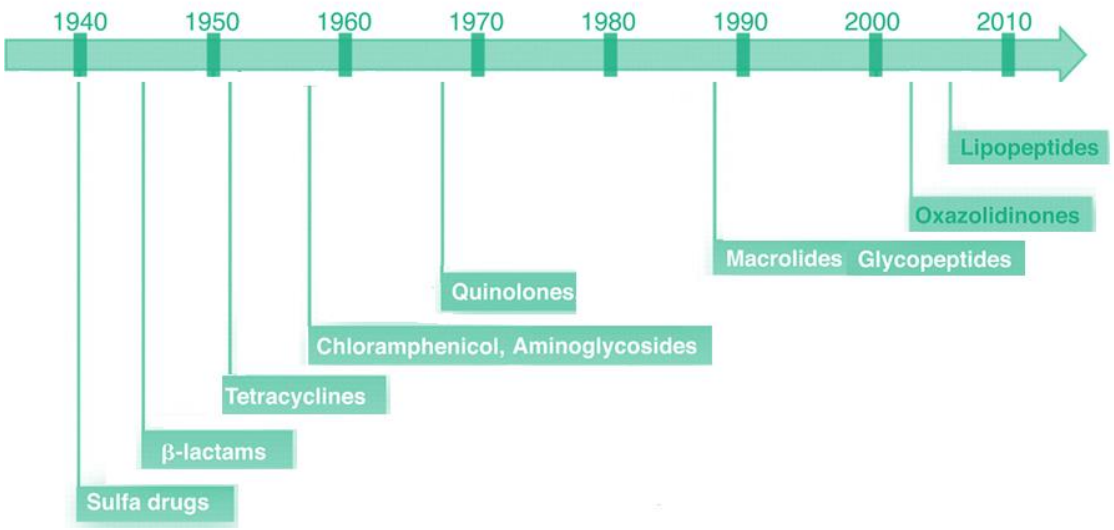


Figure 1.18 First observed antibiotic resistance

In the hospital setting where exposure to antibiotics is constant and bacterial population is numerous, a wide range of resistance mechanisms are seen. Hospital acquired (nosocomial) infections are responsible for 175000 deaths per year only in Europe, and as rates of resistance rise, death rates will also rise (86). In parallel, there has been a lack of innovation in the antibiotic discovery field, leaving potential solutions

to the rise of resistance few and far between. Pharmaceutical companies face high risks when investing in antibiotic discovery. Restricted antibiotic administration; when compared to neurological, cardiology or oncology products, translates into reduction of profits. Regulatory measures not only control the criteria used to test the efficacy of a new antibiotic but also its way of marketing. Changes adopted by FDA (Food and Drug Administration) or EMEA (European Agency for the Evaluation of Medicinal Products) have repercussions on costs and delays of clinical trials which ultimately can halt the development of a new antibiotic. Lastly, government institutions have only recognized the rising menace of antibiotic resistance during the last decade. Centers for Disease Control and Prevention (CDC), the World Health Organization (WHO) and the World Economic Forum accepted that the post-antibiotic era was no longer a distant future, but a worldwide reality that menaces public health (87). In 2014, the first global report by WHO revealed cases of resistance to 'last resort' antibiotic spread around the globe. Antibiotic resistance is a problem that affects directly or indirectly to every individual but being multifactorial requires concrete and multilateral approaches to be combated.

If a threat is to be tackled then it must be first defined. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines antibiotic resistance as the result of having a surviving population of bacteria after antibiotic exposure whose phenotypic lack of susceptibility to the antibiotic in question renders a negative clinical outcome (88). Adopting standardized methods to identify antibiotic resistance has subsenquently contributed to resistance surveillance and ultimately prioritization of strategies against the more menacing resistant pathogens (89). In 2017, the WHO priority pathogens list for research and development (R&D) of new antibiotics included three groups according to its level of resistance. In the critical level: *Acinetobacter baumanii*, *P. aeruginosa*, *Mycobacterium tuberculosis,* Enterobacteriaceae; in the high level: *Enterococcus faecium*, *S. aureus*, *Helicobacter pylori*, *Campylobacter*, *Salmonella*, *Neisseria gonorrhoeae*; and in the medium level: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Shigella* spp (90). These multidrug resistant bacteria (MDR), are often called 'superbugs' in the popular press, and belong to a larger classification separated in two groups. The first group includes those pathogens that belong to the normal human commensal flora but have acquired antibiotic resistance genes such as drug-resistant *S. aureus* and *Escherichia coli*. The second group includes those bacteria that only infect immunocompromised patients, also called opportunistic, such as *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia* that are intrinsically resistant to a wide range of antibiotics. In

Europe and also in the United States, approximately 25,000 people die each year because of a multidrug-resistant bacterial infection (91).

1.4.1 Mechanisms of resistance- β-lactamase

A penicillinase was the first β-lactamase identified in 1940. Since then more than 890 β-lactamases encoded by mobile genetic elements, plasmids and on chromosomes, have been documented. According to the amino acid sequence, the Ambler system groups these β-lactamases belong to four different classes: A, B, C, and D. Classes A, C and D are serine-β-lactamases (SBL) whose hydrolytic activity on β-lactams depends on the acylation of serine residue in the active site. Class B groups metallo-β-lactamases (MBL) whose hydrolytic activity involves the participation of zinc (92).

1.4.1.1 Serine- β-lactamases

1.4.1.1.1 Classification

Class A SBLs include penicillinases, extended spectrum β-lactamases (ESBLs) and carbapenemases. Penicillinases such as the sulfhydryl variant (SHV) in *Klebsiella pneumoniae* and the temoniera enzyme (TEM) in *E. coli, H. influenza* and *N. gonorrheae* are narrow spectrum and susceptible to long-established β-lactamase inhibitors such as clavulanic acid. Extended spectrum β-lactamases (ESBLs) were first identified as variants of SHV and TEM in *E. coli* and *K. pneumoniae*, but they have been extensively replaced by cefotaxime β-lactamases (CTX-M) in *E. coli* and *Salmonella* spp. ESBLs hydrolyze penicillins, cephalosporins, monobactams but not carbapenems. Class A carbapenamases comprise *K. pneumoniae* carbapenemase (KPC), and imipenem-hydrolyzing β-lactamases (IMI), Serratia marcescens enzyme (SME), Guiana extended-spectrum β-lactamase (GES), and non-metallo carbapenemase of class A (NMC-A). These carbapenamases have the ability to hydrolyze the last resort β-lactams (92, 93).

Class C SBLs are predominantly cephalosporinases that hydrolyse, third-generation cephalosporins such as ceftazidime, cefotaxime and the cephamycin cefoxitin. Class C cephalosporinases are normally chromosomally encoded and produced at low basal levels; however, the presence of inducing agents such as amoxicillin or clavulanic acid raise the levels of production (in some species) and make them capable of conferring resistance to other β-lactams that they do not hydrolyse well. Isolates capable of constitutively expressing AmpC enzyme in absence

of a β-lactam inducer, carry stable regulatory mutations. Chromosomal class C cephalosporinases such as AmpC are present in *Citrobacter freundii, E. coli, Enterobacter cloacae, Morganella morganii, Aeromonas* species and *P. aeruginosa*. In enterobacteria, plasmid-encoded class C cephalosporinases can be present in strains with other types of β-lactamases and can also be inducible upon β-lactam challenge. An example of a plasmid-encoded class C cephalosporinase is cephamycinase (CMY), reported in enterobacteria (92, 94, 95).

Class D SBLs are generally referred as OXA enzymes based on their ability to hydrolyze oxacillin. OXAs have been identified in *A. baumani* and *P. aeruginosa*, including those which have carbapenemase activity. Chromosomally encoded OXA enzymes are particularly dominant in *A. baumanii* (92) (**Figure 1.19**).

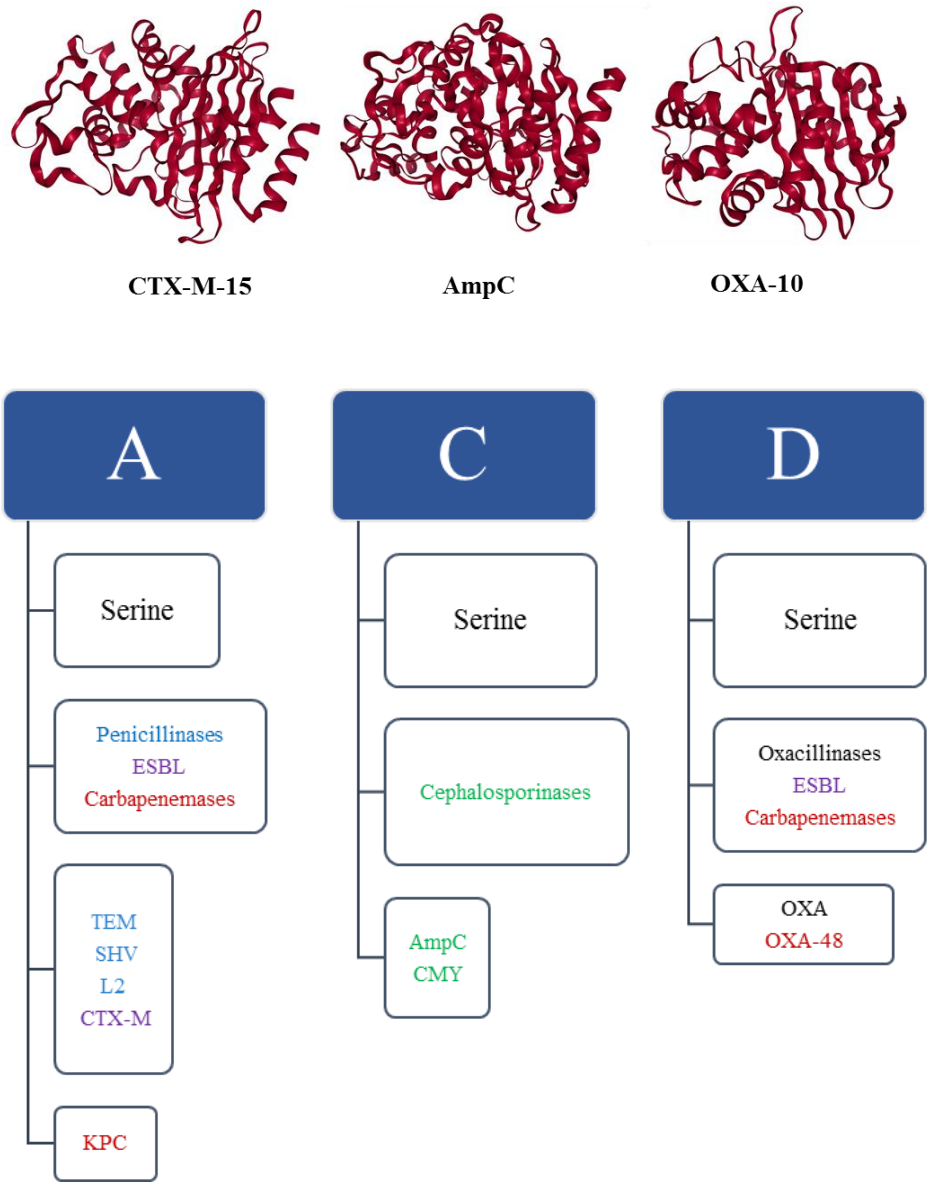


Figure 1.19 Classes of serine-β-lactamases and structural examples

Penicillinases and examples (blue), cephaloporinases and examples (green), carbapenemases and examples (red). CTX-M-15 (PDB 4S2I chain A), AmpC (PDB 1FSY chain A), OXA-10 (PDB 5FQ9 chain A). Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

1.4.1.1.2 Mechanism of action of SBLs

In general SBLs, hydrolyzes β-lactams in two steps. During the acylation step, the serine residue in the active site acts as a nucleophile to attack the β-lactam and form an enzyme-substrate acyl complex. During the deacylation reaction, a water molecule hydrolyzes the acyl enzyme complex which releases the β-lactam with an

opened β-lactam ring and the enzyme active site is regenerated. In the first step, a general base is needed to change the serine residue to a catalytic state. In the second step, a general base is required to deprotonate the water molecule during deacylation. Class A, C and D SBLs differ in the general bases they use for acylation and deacylation step (96).

Class A SBLs use Lys73 and Glu166 as general bases for acylation and deacylation, respectively. Losing Lys73 stops the reaction whilst loss of Glu166 decreases the kinetic rate. However, when the active site is at the apo-state, Lys73 is protonated and Glu166 deprotonated, which would make it unlikely for Lys73 to act on Ser70 to facilitae nucleophilic attack on the β-lactam. Quantum mechanics/molecular mechanics (QM/MM) have suggested that the mere binding of the enzyme in the apo-state with the substrate leads to changes that deprotonate Lys73, but there is no experimental evidence. Additionally, it is not entirely elucidated how the general base changes from Lys73 to Glu166 during acylation and deacylation (96). In class C SBLs, Lys67 has the same role as Lys73 in acylation whilst Tyr150 executes the role of Glu166 in deacylation. A molecule that reduces the kinetic rate of deacylation can act as an inhibitor for class C SBLs (97). Class D SBLs distinguish themselves from other SBLs by the presence of a carboxylated lysine in charge of both stages, acylation and deacylation (98) .

1.4.1.2 Metallo- β-lactamases

1.4.1.2.1 Classification

Class B β-lactamases group four subclases (B1, B2, B3 and B4), all of which are referred to as metallo-β-lactamases (MBLs) because of their use of zinc ion (s) in the active site (99). Subclasses B1 and B3 MBLs have a broad spectrum of activity, which includes penicillins, cephalosporins and carbapenems. B2 MBLs only need one zinc atom in the active site. To date, the majority of MBLs identified are in subclass B1. These enzymes include imipenamase (IMP), Verona integron-encoded metallo- β-lactamases (VIM), New Dehli metallo- β-lactamases (NDM) which are found in mobile elements (and are passed from bacterium to bacterium) in Enterobacteriaceae, *P. aeruginosa, A. baumanii, E. coli, K. pneumoniae* (73, 100).

Subclass B2 MBLs have a narrower and deeper active site when compared to B1 and B3, which might explain their poor activity against penicillins and cephalosporins and almost exclusive activity against carbapenems. The most studied B2 MBLs are chromosomally encoded CphA, or ImiS found in different species of

Aeromonas and Sfh-I in *Serratia fonticola* (101). Subclass B3 MBLsare also chromosomally encoded, the group includes enzymes such as L1 from *S. maltophilia,* GOB from *Chryseobacterium meningosepticum,* FEZ-1 from *Legionella gormanii* and the first identified mobile enzyme in the group Adelaide Imipenemase (AIM-1) in *P. aeruginosa* (101, 102) (**Figure 1.20**). Subclass B4 MBLs includes SPR-1 in *Serratia proteamaculans* which has a unique homologue in CSA-1 from *Cronobacter sakazakii* (103). Similarly to B1 and B3, B4 MBLs bind two zinc atoms in the active site (99). Within the group of β-lactams susceptible to the action of MBLs, monobactams such as aztreonam are excluded.

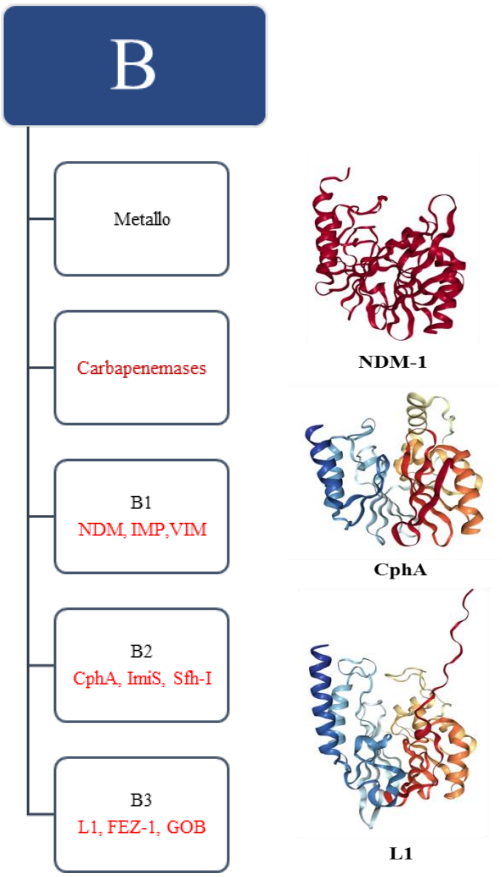


Figure 1.20 Relevant classification of metallo-β-lactamases

Metallo-carbapenamases and examples (red). NDM-1 (PDB 3SPU chainA), CphA (PDB 2GKL), L1 (PDB 1SML). Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

1.4.1.2.2 Mechanism of action of MBLs

The presence of the zinc atom is essential for the hydrolytic activity of MBLs. In B1 enzymes, the Zn1 binding site involves the participation of three histidines (His116,

His118, His196) whilst Zn2 interacts with Asp120, Cys221, and His 263 (104, 105). In class B2 MBLs the binding of Zn1 to Asn 116, His118, His196 makes it catalytically active whilst the binding of Zn2 to Asp 120, Cys 221 and His263 inhibits the enzyme (105, 106). B3 enzymes have a similar 3H site except that His116 can also be Gly116, and Zn2 binds to Asp120, His 121 and His 262.

In general, the hydrolytic mechanism of MBLs involves two steps: cleavage of the amide bond and protonation of the generated intermediate. With the substrate binding, the Michaelis complex (ES) is established and with it the positioning of the water/hydroxide molecule between the two zinc atoms. The bridging water, being a nucleophile, attacks the carbonyl carbon (C7) which leads to the cleavage of the C-N bond (an opened β-lactam ring) and formation of an anionic nitrogen intermediate stabilised by Zn atoms (EI). The intermediate becomes protonated and an EP complex is formed before the product release (105, 107).

1.4.2 β-lactamase inhibitors

The search for β-lactamase inhibitors started in the 1970s. β-lactamase inhibitors can bind irreversibly or reversibly which implies covalent or non-covalent binding to the active site, respectively. During irreversible binding, covalent interactions with β-lactamases prevent a rapid dissociation of the inhibitor, in contrast to reversible inhibition (108, 109). Reversible inhibitors are poor substrates with high affinity but low hydrolysis rates. In general, irreversible inhibitors are considered better inhibitors since they inactivate the enzyme (110).

1.4.2.1 β-lactam based inhibitors

β-lactamase inhibitors were first conceived as irreversible that mimick the binding of a β-lactam in the active site of an SBL. The first of this class to be discovered was clavulanic acid which was originally obtained from *Streptomyces clavuligerus*. Clavulanic acid has a five-membered ring, fused to a β-lactam ring, where the sulfur atom has been replaced by an oxygen atom. An acylamino side chain is absent in the β-lactam ring. Clavulanic acid is considered a suicide irreversible inhibitor since its fragmentation (β-lactam ring opening by the catalytic action of a serine residue) produces an initial acyl-enzyme intermediate that reacts with a nucleophilic group to render a long lived transient intermediate that ultimately will proceed to inactive the enzyme (32). Clavulanic acid is particularly efficient against Ambler class A penicillinases, ESBLs but not carbapenemases (**Figure 1.21**) (108).

Other β-lactam based inhibitors are tazobactam and sulbactam which are penicillinate sulfones. Both conserve the sulfur atom in the ring attached to the β-lactam ring, but tazobactam has an additional triazole group at C2 position. Sulbactam is slightly more active than clavulanic acid against cephalosporinases, but the triazole group in tazobactam improves its potency against class A and C β-lactamases. In fact, tazobactam inhibits P99 cephaloporinase from *Enterobacter cloacae* with less than 20 turnovers of inhibitor per enzyme. The number of turnovers before the inactivation of the enzyme determines the potency (110). Additional experimental β-lactam based inhibitors include tazobactam analogues such as AAI101, penam sulfones such as LN-1-255, sulbactam analogues such as BAL19764 and bridged monobactams such as BAL29880 (111).

1.4.2.2 Non-β-lactam based inhibitors

Diazabicyclooctanones (DBOs) non-β-lactam based inhibitors whose structures were inspired in anti-Bredt bicyclo γ-lactams which were originally obtained to inhibit β-lactamases and PBPs but required structural optimisation (112, 113). Avibactam was the first of the series to be approved for use in clinics. What is particularly interesting about avibactam is its unique mode of binding, covalent reversible binding from where dissociation of the enzyme releases intact avibactam. Avibactam has nanomolar affinity for class A, C and some D SBLs and it has gained approval to be combined with ceftazidime, and soon with ceftaroline or aztreonam to restore antimicrobial activity against Gram-negative bacteria (**Figure 1.21**). Relebactam is an avibactam analogue that has an additional piperidine group in the DBO ring and similar activity against β-lactamases except for the class D carbapenemases OXA-48 (108, 114). Nacubactam has a carboxamide side chain which inhibits SBLs as well as PBP2. ETX2514 is still in pre-clinical development and contains a substitution in the piperidine ring which confers more reactivity and diffusion through the cell membrane (111).

Boronic acid based compounds have been known as inhibitor for serine enzymes for decades. Acyclic boronates inhibit human serine hydrolases, however the active site of SBLs is wider and can accommodate cyclic boronates. Vaborbactam was the first in the series to be approved for clinical use for its nanomolar affinity towards Ambler class A, C and D SBLs. Optimisation to obtain a SBL/MBL inhibitor led to RPX-7282, which exhibits good activity against all β-lactamases. Most recently, bicyclic boronates have been found to be another class of pan-β-lactamase inhibitors, which also inhibit PBPs (**Figure 1.21**) (111).

1.4.2.3 Zinc-binding inhibitors

Sulfur based compounds provided the first hint to design MBL inhibitors since zinc binds to the sulphur atom. Thiomandelic acid was the first to show micromolar affinities for a broad range of MBLs (**Figure 1.21**). The azolythioacetamide provided a scaffold for triazolylthioacetamide that inhibit the important class B1 MBL, NDM-1. Thiol ester derivatives inhibit the class B3 MBL L1 and release mercaptoacetic acid which were the first inhibitors obtained based on the mechanism of inhibition and are active against NDM-1 and L1. L- and D- captopril contain thiol and carboxylate groups capable of forming coordination bonds with the zinc atoms in the binding site of all MBL subclasses. Thiazolidine derivatives such as bisthiazolidines share homology with β-lactams and inhibit all MBL subclasses through different modes of binding (**Figure 1.21**) (115).

Other attempts to obtain zinc-binding inhibitors include carboxylate or phosphonate groups, derivatives from benzoic acid, dipicolinic acid, maleic acid, and more recently triazole (111).

1.4.2.4 Zinc-chelating inhibitors

An alternative form to design inhibitors is to hijack the zinc atom required for the mechanism of action of MBLs. EDTA is effective but unspecific, especially when considering that human metallo-enzymes often also require zinc. Aspergillomarasmine A derives has four carboxylate groups that sequester the zinc atoms. Similarly, spiro-indoline-thiadiazole and macrocyles such as NOTA and DOTA have been shown to chelate zinc atoms to restore carbapenem activity against MBL producing bacteria (**Figure 1.21**) (111).

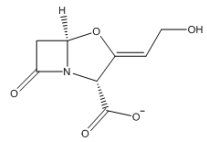
1.4.2.5 Covalent inhibitors

Covalent inhibition has been reported for urease activity in the presence of β-mercaptoethanol and 1, 4 benzoquinone. Ebselen is a selenium containing compound that binds with the class B1 MBL, NDM-1 due to the opening of the selenazol ring (**Figure 1.21**).

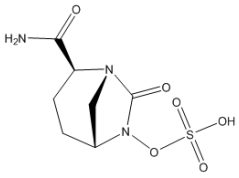
The search of a universal β-lactamase inhibitor is limited due to tigh profile that these inhibitors need to fulfil such as cellular uptake, low cytotoxicity, *in vitro* efficacy, nanomolar affinity, to name a few (111).

β-lactamase based inhibitors

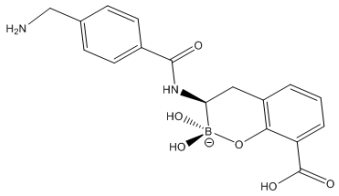
Non-β-lactamase based inhibitors



Clavulanic Acid

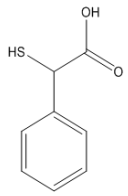


Avibactam

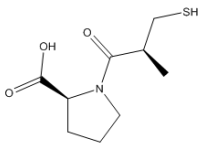


Bicyclic boronate

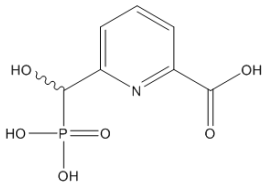
Zinc-binding inhibitors



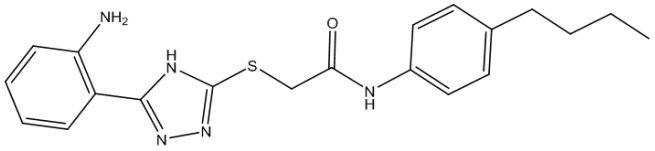
Thiomandelic acid



L-captopril

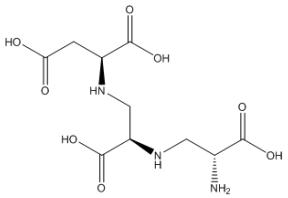


Phosphonate-based 3b

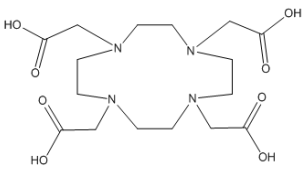


Triazole-based

Zinc-chelating inhibitors

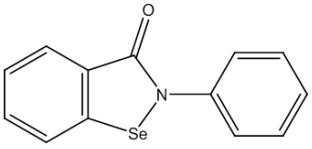


Aspergillomarasmine A



DOTA

Covalent inhibitors



Ebselen

Figure 1.21 Representatives of β-lactamase inhibitors

1.4.3 Mechanisms of resistance - Aminoglycoside modifying enzymes

Aminoglycosides can be modified by the activity of acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs), and nucleotidyltransferases (ANTs). AACs acetylate -NH_2 groups in the 2-deoxystreptamine or the sugar groups by using acetyl coenzyme A (**Figure 1.22**). Acetylation can occur at positions: 1 [AAC (1)], 2' [AAC (2')], 3[AAC(3)], or 6'[AAC(6')] (116).

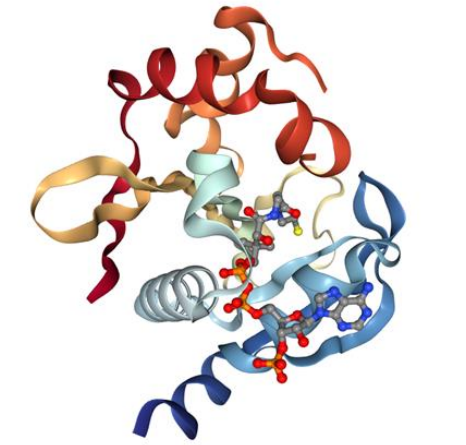


Figure 1.22 AAC(6')-Ib in complex with CoA (PDB 2PRB)

Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

Aminoglycosides molecules can also be altered by APH phosphorylation. Depending on where the modification occurs, classes of APHs include: APH (4), APH (6), APH (9), APH (3') (**Figure 1.23**). APH (2''), APH (3'') and APH (7'') (116).

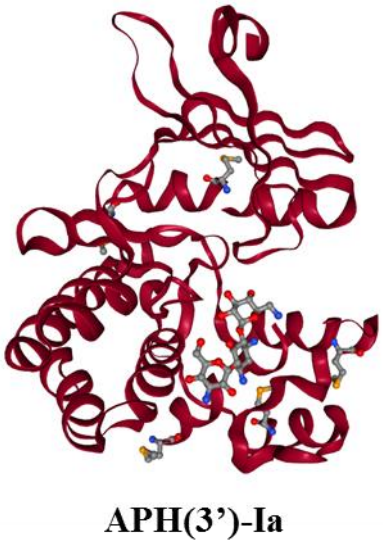


Figure 1.23 APH(3') Ia complexed with kanamycin (PDB 4GKH)

Structure displays seleno-methionene residues. Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

1.4.4 Mechanisms of resistance - reduced antibiotic penetration into cell

The outer membrane of Gram-negative bacteria contains a bilayer of lipopolysaccharides and phospholipids where specific water-filled uptake channels named porins and ligand-gated porins are embedded. While alterations in the number or type of porins in the outer membrane can reduce the rate of entry of antibiotic, inner membrane multidrug efflux transporters, (including major facilitator superfamily transporters, resistance-nodulation division type efflux pumps, ATP-binding cassette or ABC transporters) can reduce the antibiotic concentration in the cell by their active expelling from the cytoplasm to the exterior (117).

1.4.4.1 Major Facilitator Superfamily (MFS) efflux pumps

Active antibiotic efflux by MFS antiporters is attributed to their capacity of using the potential energy stored in a chemical gradient across the cytoplasmic membrane pump substrate against a concentration gradient (118). MFS transporters consist of 12

transmembrane spanning segments (TMS), grouped in a N-terminal and C-terminal domain, and an aqueous cavity between the two domains (119) (**Figure 1.24**). Based on this structure, it has been determined that the substrate-binding site originally faces one side of the membrane but after binding the conformation of the site alters, to present it to the other side (120). In Gram-negative bacteria, MFS transporters work as a monomer or in association with membrane fusion proteins and, outer membrane proteins as a so-called 'tripartite' pump to transport antibiotics not only to the periplasm but to the exterior of the cell (121).

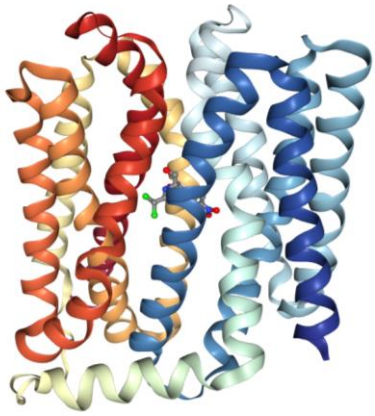


Figure 1.24 Example of MFS efflux pump complexed with chloramphenicol

Structural representation of MdfA MFS exporter in *E. coli* (PDB 4ZOW). Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

1.4.4.2 Resistance Nodulation *Division (RND) type antibiotic efflux pumps*

RND efflux pumps are located in the inner membrane and their drug efflux function relies on the proton motive force. They are connected to a periplasmic protein adaptor, known as a membrane fusion protein (MFP) and so to an outer membrane protein (OMP). These three proteins work as a tripartite complex that spans the cell envelope (122) (**Figure 1.25**).

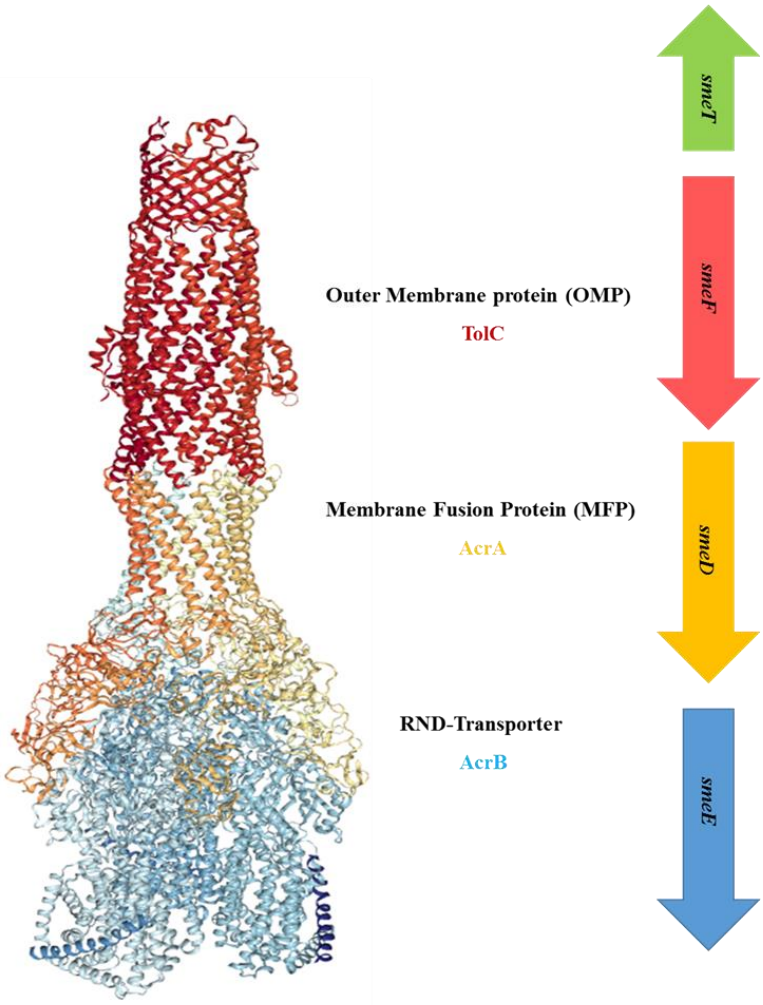


Figure 1.25 RND-Transporter

Structural representation of AcrAB-TolC (PDB 5V5S) RND efflux pump. Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

1.4.4.3 ATP Binding Cassette Superfamily (ABC) efflux pumps

ABC-type efflux pump proteins, contain two domains for ATP binding and two transmembrane domains (**Figure 1.26**). The ATP binding domains, signatures of ABC transporters, are well conserved in prokaryotes and eukaryotes. Key domains, Walker A and B, are involved in ATP hydrolysis and Mg^{2+} ion coordination. After ATP hydrolysis, conformational changes in the transmembrane domains allows transport across the membrane (123, 124).

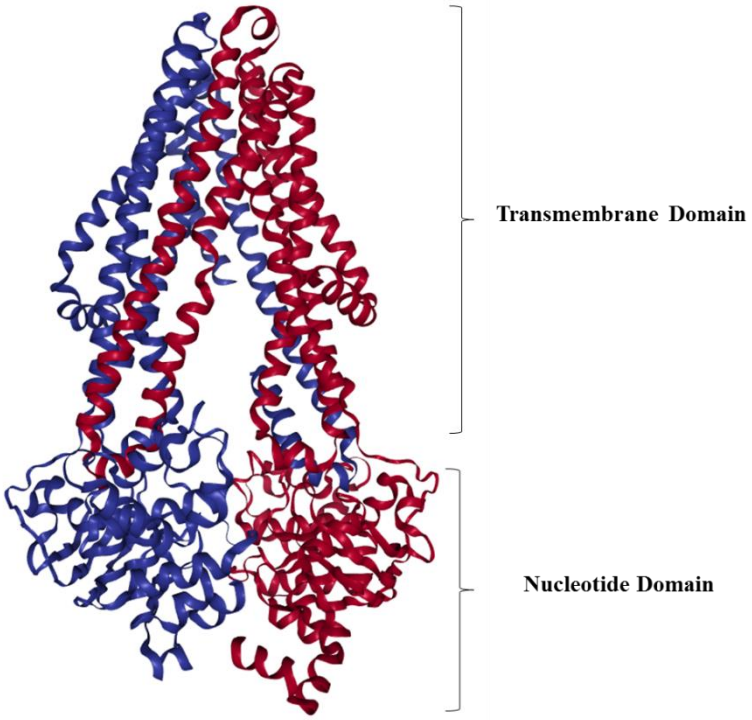


Figure 1.26 ABC transporter

Structural representation of MsbA ABC transporter in *E. coli* (PDB 6BPL). Molecular visualization with NGL viewer from the set of tools from Galaxy platform (https://usegalaxy.org/)

1.4.5 Other mechanisms of resistance

1.4.5.1 Target modification and protection

Target site modification or protection is not as prevalent as mechanism that alter or prevent entry of the drug, but two good examples that reveal the complexity are aminoglycoside and tetracycline resistance. These antibiotic classes prevent protein synthesis by impeding the binding of tRNAs to the ribosome in the A-site. To compensate this effect, bacteria can either reduce influx, increase efflux, modify the antibiotic or overexpress the target (125). In fact, aminoglycoside resistance for example can arise from mutations occurring in the 16S rRNA where the aminoglycoside binding occurs or in ribosomal proteins, important for antibiotic interaction, such as *rpsL*. Alternatively overexpression of the 16S rRNA can hijack the drug which leaves unhibited target molecules (125) . Aminoglycoside efficacy can also

be altered by methylation of the binding site due to the action of 16S ribosomal methyltransferases (126).

Tetracycline resistance occurs due to the ribosomal assisted protection which include TetM and TetO that act on the ribosome-tetracycline complexes to remove the antibiotic (127, 128). Similarly, pentapeptide repeat proteins (PRPs) bind to quinolone-toporisomerase complex to release the antibiotic fron the binding site (129).

1.5 Overview of *S. maltophilia*

The ability of *S. maltophilia* to survive on humid surfaces increases its chances of colonising medical devices that breach the natural barriers of immunity in patients, such as ventilators, nebulizers, and catheters (130). Consequently, it is not surprising that *S. maltophilia* infections are mainly ventilator associated lower respiratory tract infections and catheter related, and bloodstream infections. It also causes, skin and soft tissue, bone and joint and urinary tract infections. *S. maltophilia* also causes an increasing number of infections but also in cystic fibrosis patients (130).

The first record of the bacterium now known as *S. maltophilia* is attributed to a specimen isolated from pleural fluid in 1943 in the UK. Originally, the isolate was named '*Bacterium bookeri* NCTC 6572' but further morphological, physiological and serological characterisation demonstrated that '*Pseudomonas maltophilia*' was a better designation to describe a group of microorganisms that fulfil certain biochemical and physiological attributes such as strict aerobic growth, yellow pigmented colonies, maltose metabolism, and uni, bi or multi-flagellated (131). Later, comparative biochemical and molecular biological characterisation suggested a transfer of *P. maltophilia* to the genus *Xanthomonas*. However, results based on 16S RFLP led to *Stenotrophomonas* emerging as a completely new genus. The genus belongs to the Gammaproteobacteria class and includes eight species: *S. maltophilia, S. nitritireducens, S. rhizophila, S. acidaminiphila, S. koreensis, S. chelatiphaga, S. terrae* and *S. humi.* The only species known to be a human pathogen is *S. maltophilia* (132). In fact, *S. maltophilia* pathogenesis might respond to its environmental niche, which makes it a ubiquitous organism.

1.5.1 Prevalence and clinical relevance

The most susceptible population to *S. maltophilia* infections are debilitated patients with weakened immunity. Risk factors for infection include hematological

malignancy, organ transplantation, HIV infection, cystic fibrosis, extended hospitalization, intensive care unit stay, mechanical ventilation, indwelling catheters, therapy with immunosuppresors and previous antibiotic therapy (131). However, *S. maltophilia* infections are not limited to hospitalized patients. Community infections were frequently associated with at least one of the risk factors mentioned above (133).

The first report of *S. maltophilia* as a human pathogen was in 1980. Since that time, its isolation has become more frequent, especially in patients with neutropenia after exposure to chemotherapy and patients with indwelling catheters. In 1990, surveillance programs started to provide information about the prevalence of *S. maltophilia.* First locally such as the Canadian Ward Surveillance Study (CANWARD), the British Society for Antimicrobial Chemotherapy (BSAC) Resistance Surveillance Project, the Taiwan Surveillance of Antimicrobial Resistance (TSAR) and then global surveillance programs such as the SENTRY Antimicrobial Surveillance Program launched in 1997 and the Study for Monitoring Antimicrobial Resistance Trends (SMART), in 2002 (134). However, the closest estimation of the actual prevalence of *S. maltophilia* as a cause of infection in the general population comes from comparison of large surveillance studies over time. From 1997-2003 the prevalence of *S. maltophilia* infection in the general population, globally, increased from 0.8-1.4% and from 1.3-1.68% during the period 2007-2012 (135, 136). It is not surprising that the prevalence in intensive care units is higher than in general population (1.4-3%) (134). Until 2004, SENTRY reported 1.2% prevalence as a cause of infection in children ≤ 7 years and 1.4% in children ≤ 18 years. According to WHO *S. maltophilia* is classified as a leading multidrug-resistant organism in hospital settings (nosocomial infection) (137). Attributable mortality to *S. maltophilia* infections ranges from 21-69% that can reduce when the initial drug therapy is adequate (138, 139).

Aside from bacteraemia and pneumonia, *S. maltophilia* is also associated with biliary sepsis, infections of the bones and joints, urinary tract and soft tissues, eye infections, endocarditis, meningitis, anatomic abnormalities and surgical manipulation (140). As stated before, *S. maltophilia* is a cystic fibrosis (CF) associated pathogen whose prevalence in CF patients increases with age. The CF Foundation Patient Registry (CFFPR) determined a remarkable increase in *S. maltophilia* prevalence from 1988-2012. This can be attributable to an improved methodology in diagnosis and report in microbiology laboratories but also to the extended lifespan, long-term antimicrobial exposure, and possibly person-person transmission. However, *S. maltophilia* infections in CF patients seem to be acquired from non-healthcare-associated environmental sources or from long-term antimicrobial

exposure. Since *S. maltophilia* can be found in natural environments, it is advisable for CF patient to avoid prolonged exposure to soil, and not appropriately chlorinated pools or stagnant water. The healthcare sources associated to *S. maltophilia* in CF patients are not totally understood but in non-CF patients *S. maltophilia* infections have been linked to water sources (sink drains, faucets, potable water) and use of moisturizing lotion, instead of soap, by healthcare staff (141).

1.5.2 Diagnosis

Standard techniques allow diagnosis of *S. maltophilia*. However, culture based identification can be prone to error and is time consuming. For example, hematological diagnosis of *S. maltophilia* takes 4.5 days including the result from blood culture (142). When a patient has sepsis every hour of delay in the diagnosis represents a 7% increase in mortality (143). Since the determination of the genome sequence of *S. maltophilia* strain K279a faster and more accurate methods of diagnosis have been developed (144). A 5-plex real-time PCR nucleic acid diagnostics assay (NAD) was designed to identify and quantify the presence of *S. maltophilia* in water sources but demonstrated to be an assay applicable in clinics (145). A loop-mediated isothermal amplification (LAMP) method allowed to detect the L1 metallo-β-lactamase, with little amounts of DNA and within one hour (146). Target enriched multiplex PCR (TEM-PCR^{TM}) can be used to detect *S. maltophilia* in a bloodstream infection. A peptide nucleic acid (PNA) probe allows *S. maltophilia* identification using fluorescence *in situ* hybridization (FISH) within 90 minutes with 100% sensitivity and specificity on pure culture and sputum samples from cystic fibrosis patients (147). Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-ToF MS) has enabled identification of non-fermenting Gram-negative bacteria, including *S. maltophilia* (148). In fact in reference units such as antimicrobial resistance and healthcare associated infections reference unit (AMRHAI) in the UK, MALDI-ToF MS and sequence-based identification is offered for a presumed *S. maltophilia* strain (149).

1.5.3 Treatment

S. maltophilia has gained its reputation due to the limited options in treatment. The combination of trimethoprim and sulfamethoxazole (TMP-SXT), co-trimoxazole, is frequently the first antibiotic choice against *S. maltophilia* infections. The course of antibiotic administration normally occurs during nine days (142). TMP-SXT inhibits the

synthesis of tetrahydrofolic acid, which is needed for the synthesis of thymidine, purines, and bacterial DNA. On the one hand, sulfamethoxazole, a para-aminobenzoic acid (PABA) analogue prevents the synthesis of one of the precursors of tetrahydrofolic acid, dihydropteroic acid by inhibition of dihydropteroate synthase. On the other hand, trimethoprim, a pteridine analogue, prevents the reduction of dihydrofolate to tetrahydrofolate by inhibition of dihydrofolate reductase. Abolishing two steps in one metabolic pathway leads to a bactericidal activity (150). However, side effects such as allergic reactions and concentration-dependent toxicity, and the presence of resistance mechanisms limit its use in *S. maltophilia* (151).

The Clinical and Laboratory Standards Institute make available guidelines to test alternative antimicrobial agents against *S. maltophilia* using disc diffusion and microtitre both dilution (MIC methodology). Apart from co-trimoxazole, chemotherapeutic options against *S. maltophilia* infections include monotherapy or combination of the following drugs: ceftazidime, ticarcillin-clavulanate, minocycline, tigecycline, chloramphenicol, polymyxins, ciprofloxacin, and levofloxacin (135, 152). Although some of them exhibit good in vitro activity, reported success in clinical settings is limited (135).

1.6 Antibiotic Resistance Mechanisms in *S. maltophilia*

The sequenced genome of a representative Group A strain of *S. maltophilia*, K279a, identifies the presence of several elements to which intrinsic antimicrobial drug resistance is attributed. *S. maltophilia* phylogenetic Group A includes homogenous isolates in terms of their 16s rRNA and *smeT-smeD* intergenic region sequences (153). These resistance mechanisms include: limiting the entrance of antibiotic by permeability alterations in the cell envelope, active efflux of the antibiotic once it is in the cell, enzymatic mechanisms that destroy or modify the drug that prevents the action of the antibiotic (154). For example, the low overall outer-membrane permeability, due to the low abundance of porins, interferes with the drug cellular uptake. Likewise, the presence of nine RND-efflux pumps (most notably SmeDEF, SmeYZ and SmeIJK) confers resistance to fluoroquinolones, chloramphenicol, aminoglycosides and tetracycline due to drug extrusion, when these pumps are over-produced following mutation. Modifying enzymes, such as the class B3 MBL L1 and the class A SBL L2, confer resistance to most and in some cases all β-lactam options (155).

1.6.1 β-lactam resistance in *S. maltophilia*

1.6.1.1 β-lactamase production

S. maltophilia has two chromosomally encoded β-lactamases. L2 is an SBL that belongs to the ESBL subclass of the Ambler class A (156). L1 is a tetrameric MBL that belongs to Ambler subclass B3 (157) . L1 and L2 both confer resistance to cephalosporins and penicillins, and uniquely to carbapenems in the case of L1 and monobactams in the case of L2 (158).

In order to exert their hydrolytic activity on these β-lactams, mature β-lactamases must be translocated from the cytoplasm to the periplasm. The systems involved in this process are Sec and Tat. While the Sec system exports unfolded proteins, the Tat system transfers folded proteins. Before translocation, immature eproteins contain a signal peptide that contains 24 amino acids on average and once in the periplasm is cleaved. In *S. matophilia,* L2 is Tat dependent and L1 is exported by Sec system (159). Despite having different export systems, expression of both β-lactamases shares some regulatory elements, such as the transcriptional activator, AmpR, that belongs to the *ampR-L2* module (160). L1 and L1 production can be induced in the presence of a β-lactam or hyperproduced as a result of mutations in the regulatory circuit that controls induction.

The major mechanisms involved in β-lactamase induction is the AmpG-AmpR-AmpC pathway (26) (**Figure 1.27**). In *S. maltophilia* as in other Gram-negatives, during cell growth and division, lytic tranglycosylase enzymes (LT) cleave the glycan strand between NAM and NAG, and form an exposed anhydro-NAM and an endopeptidase breaks the cross-linking between stem oligopeptides. Then anhydro muropeptide monomers (NAG-anhydro-NAM-peptides) are liberated and transported into the cytoplasm through the inner membrane transporter AmpG where NAG is removed by the glycoside hydrolase NagZ. The final products, 1,6-anhydroNAM-oligopeptides, are transformed by a series of enzymes to UDP-NAM-pentapeptides which are PG precursors that can be recycled into the PG biosynthesis pathway, but also they can be sensed by the transcriptional regulator AmpR to induce the production of β-lactamase (26).β-lactams activate β-lactamase production by increasing the breakdown of PG, and so the abundance of the AmpR activatory ligand. A negative effector that reduces the expression of β-lactamase is the amidase AmpD which can remove stem peptides from anhydro-NAM thus reducing the concentration of the AmpR activator ligand during growth in the absence of β-lactams (26).

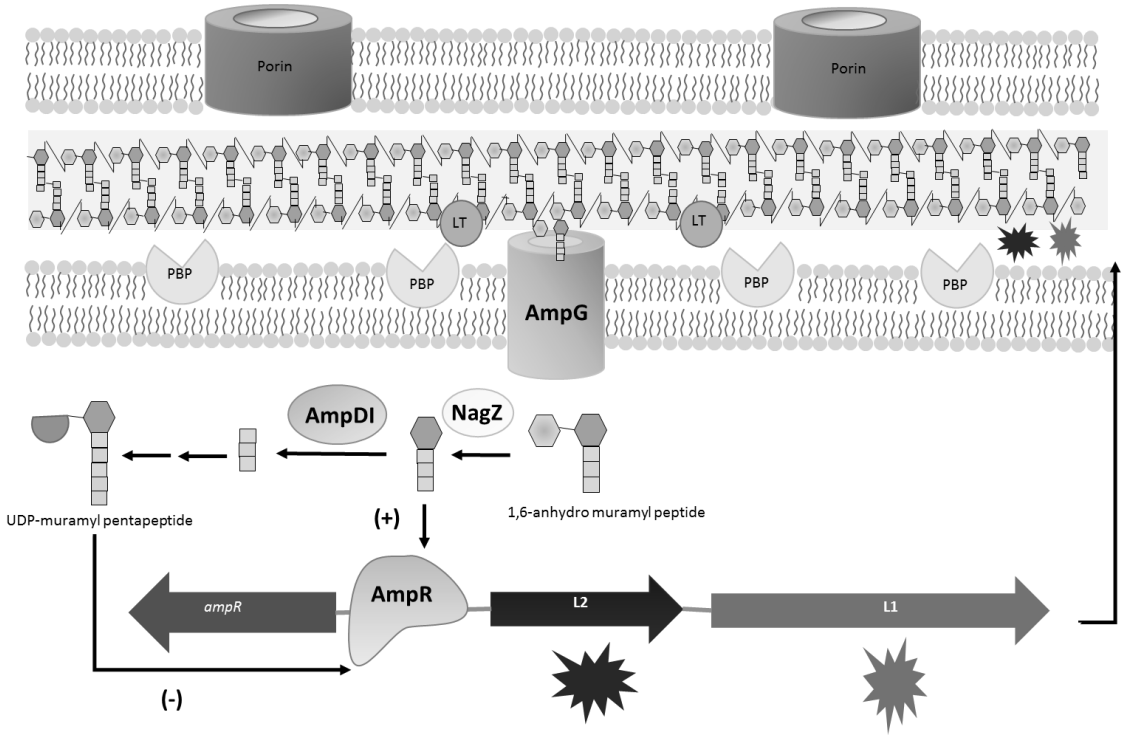


Figure 1.27 β-lactamase production via AmpG-AmpR

Muropeptides (GlcNAc-1, 6 -anhydroMurNac tri, tetra, and pentapeptides are recycled from the periplasm through AmpG where NagZ removes GlcNAc. Then, the NagZ-processed product can induce β-lactamase (AmpC in Enterobacteria and *P.aeruginosa* or L2 in *S. maltophilia*) production by binding to AmpR, or be further processed by AmpD that excises the peptides from GlcNAc-1,6-anhydro-MurNAc and 1, 6-anhydroMurNac. PG degraded products will then be recycled as UDP-MurNAc peptides which act as repressor ligands of AmpR. Inactivation of the enzymes involved or β lactam exposure cause accumulation of degraded products that activate AmpR.

As well as being more commonly attributed to an AmpR mediated mechanism, induction of β-lactamase production and so β-lactam resistance has also been attributed to the two-component regulatory system (TCRS) in some bacteria (26). Recently, proteomics analysis revealed the increase of two members of different TCRS in *S. maltophilia* after imipenem challenge: and integral membrane sensor histidine kinase (Smlt3765) and a GGDEF response regulator (Smlt4295) (161). However, the role of TCRS during β-lactamase induction in *S. maltophilia* is still uncertain and needs further research.

1.6.2 Quinolone resistance in *S. maltophilia*

1.6.2.1 Smqnr gene

Smqnr is a chromosomally-encoded pentapeptide repeat protein that is related to the Qnr proteins encoded on mobile genetic elements in Enterobacteriaceae (162-164). Qnr proteins share some similarity to proteins involved in DNA-replication protection such as McbG and MfpA. These proteins are DNA mimics that bind to the DNA gyrase and topoisomerase IV. Similarly, it is believed that Qnr binds to the region of these topoisoemerases that are the quinolone target sites, thus protecting the targets from antimicrobial activity (64, 165).

1.6.2.2 SmrA (ABC transporter)

SmrA was the first ABC transporter identified in *S. maltophilia*. SmrA has been found to have 70.8 % similarity with VcaM from *Vibrio cholerae*. SmrA and VcaM are half-transporters since they have two fused homodimeric subunits. Although the regulator for SmrA is unknown, its role it quinolone resistance has been demonstrated by expression in a heterologous host*, E. coli* (164, 166). It is usual to find ABC transporters regulated by two-component systems (TCSs) which are normally encoded contiguously to the transporter(167).

1.6.2.3 SmeDEF (RND efflux pump)

A major determinant for quinolone resistance in *S. maltophilia* is the overexproduction of the RND efflux pump, SmeDEF, which is encoded by an operon. SmeD is an AcrA homologue, found in *E. coli* which is an MFP whilst SmeE shows 61% homology to the RND efflux protein AcrB (**Figure 1.25**), in *E. coli*. AcrA contains four domains: α-helical, lipoyl, β-barrel and membrane-proximal. SmeE is predicted to have 12 TMS as AcrB does, and two periplasmic loops (168). In AcrB, the periplasmic portion contains a porter and a funnel domains. The porter domain, closer to the inner membrane, contains two substrate-binding pockets composed of aromatic, polar and charged amino-acids that facilitates the interaction of a broad range of substrates. During substrate-binding, AcrB changes its conformation due to protonation of the structural repeats of the trans-membrane. Thus, only drug efflux is allowed and its returning is not possible (169).

1.6.3 Tetracycline resistance in *S. maltophilia*

1.6.3.1 SmtcrA (MFS transporter)

S. maltophilia posses a bicistronic operon that encodes SmqnrR (DeoR-type regulator) and SmtcrA (MFS transporter). This operon is located upstream of the Smqnr quinolone resistance gene. In some strains, deletion of the repressor regulator increases SmtcrA production, which is responsible for tetracycline resistance. However, the effect of the regulator deletion on quinolone resistance due to Smqnr expression might be strain-specific (170, 171).

1.6.3.2 SmeDEF and SmeIJK (RND efflux pumps)

Resistance to tetracycline is mainly attributed to tefflux by SmeDEF and SmeIJK. SmeIJK is particularly interesting since the MFP SmeI can form a complex with the RND transporters SmeJ or SmeK. While the overexpression of *smeIJK* confers tetracycline resistance, disruption of either *smeJ* or *smeK* restores susceptibility to some extent (172, 173).

1.6.3.3 SmrA (ABC transporter)

SmrA not only confers resistance to quinolones but also tetracyclines. However, the resistant phenotype has only been described for laboratory strains and not for clinical isolates. Later, expression of *smrA* was assessed in a collection of 450 *S. maltophilia* isolates. Here, levels of *smrA* expression between tigecycline susceptible and non-susceptible strains did not reveal significant differences. Instead, tigecycline resistance was attributed to SmeDEF (174).

1.6.4 Aminoglycoside resistance in *S. maltophilia*

1.6.4.1 Aminoglycoside modifying enzymes

In *S. maltophilia*, putative AAC (2') and AAC (6')-Iz are cromosomally encoded. In general, the AAC (6')-I class is mainly active against amikacin but some variants can also act on gentamicin and quinolones. Despite low expression of AAC (6')-Iz, this enzyme is capable of contributing to confer resistance to amikacin (175). Other variants such as AAC (6')-Ib can accommodate structurally different substrates due to the plasticity of its active site (176, 177). *S. maltophilia* has a cromosomally encoded APH (3')-IIc which confers resistance to kanamycin (176, 178).

1.6.4.2 SmeIJK, SmeOP, SmeYZ (RND efflux pumps)

Aminoglycosides are a substrate for many RND trasnporters including SmeABC, SmeIJK, SmeOP, and SmeYZ. SmeOP-TolCSm is regulated by the upstream located repressor, SmeRo. Disruption of *smeOP* or *smeRo* increase the level of susceptibility to aminoglycosides (179). Likewise, a role in aminoglycoside resistance has been attributed to SmeYZ overexpression. Again, regulation of *smeYZ* expression is controlled by a two-component system, SmeSyRy (180).

1.6.4.3 MacABCsm (ABC transporter)

The ABC transporter, MacABCsm, present in *S. maltophilia* contributes to aminoglycoside resistance. MacABCsm is constitutively expressed and besides its role in antimicrobial resistance posesses physiological roles related to cell protection during envelope and oxidative stress (181).

1.6.5 Horizontal gene transfer

Integrative and conjugative elements such as transposons, integrons, plasmids, and genomic islands contribute to the horizontal spread of acquired resistance (182). In *S. maltophilia,* resistance to the TMP-SXT combination, which is the drug of choice is the result of a class 1 integron containing *sul1* gene (130, 182). In addition, a high-level of ampicillin resistance has been attributed to the presence of the gene bla_{TEM} in a Tn*1*/Tn*3*-type transposon in one clinical isolate (183). However, the low frequency of finding integrons and plasmids would suggest that they are not the major mechanisms for the dissemination of antibiotic resistance in *S. maltophilia* (184). Which, as discussed above, relies on intrinsic resistance mechanisms, and mutations that upregulate them for the few antimicrobials that have clinical efficacy.

1.7 Aims of the project

S. maltophilia is an emerging and relatively poorly studied pathogen, the overall am of the work reported in this thesis was to better characterise mechanisms of antibacterial drug resitance and to test the ability of novel antibacterials and inhibitors of resistance to kill *S. maltophilia.* Specifically the objectives were:

To follow up previous work from the groups that concluded that there are at least two uncharacterised mechanisms of ceftazidime resistance: one novel mechanism causing β-lactamase hyperproduction and one that does not involve β-lactamase hyperproduction. We set out to identify and characterise these mechanisms.

To follow up on previous work from the group reporting SmeYZ efflux pump mediated aminoglycoside resistance and SmeDEF efflux pump mediated fluoroquinolone resistance by identifying the reasons for SmeYZ or SmeDEF overproduction in each case. Also to select and characterise the mechanisms used by mutants resitant to minocycline and levofloxacin since ther are considered valid therapies when co-trimoxazole cannot be administered.

To evaluate the activity of various β-lactamase inhibitors in combination with various β-lactams against highly drug resistant *S. maltophilia* mutants and clinical isolates and to identify the spectrum and mode of activity of, and mechanisms of resitance to these combinations

To evaluate the antimicrobial activity of chemically modified lactivicin antibiotics against a collection of *S. maltophilia* clinical isolates, and to evaluate mechanism of reduced susceptibility to these agents.

2 Materials and Methods

2.1 Bacterial isolates and materials

S. maltophilia clinical isolates here used originated from the SENTRY antimicrobial resistance survey and have been previously described (185, 186) or were isolated from patients being treated at the Bristol Oncology Centre (183). Isolates of other species were either obtained from type strain collections, or were clinical isolates collected by SENTRY and gifted by Dr Mark Toleman, Cardiff University, or have previously been described (187). The ceftazidime resistant, β-lactamase hyper-producing mutants K CAZ 10 (188), KCAZ14 (188), KM11 (160), have previously been described. Mutants K AMI 8, K AMI 32, K MOX 2 and K MOX 8 were selected using K279a as parent strain as described previously (189). All growth media were from Oxoid. Chemicals were from Sigma, unless otherwise stated. LTV13 was re synthesized according to the literature protocol and kindly provided by Prof. C. Schofield, University of Oxford (190). LTV17 was kindly supplied by Pfizer. Avibactam was supplied by AstraZeneca whilst cyclic boronate 2 was synthesised according to published protocols (191) and provided by Prof. C. Schofield, University of Oxford. Likewise hydroxylcoumarin cephalosporin, FC5, (192) has been previously described and was provided by Prof. C. Schofield, University of Oxford. Synthesis of PMPC-1 (3a) has been reported previously (193) and provided by Dr. Gary I. Dmitrienko, University of Waterloo. *E. coli* strains such as DH5α chemically competent cells (ThermoFisher) were used in DNA manipulation experiments while SoluBL21 (DE3) cells (Gentlantis) were used for protein expression.

2.2 Primers and plasmids

Primers used in this study are described in **Table 2.1** Plasmid maps are described in Figure 2.1 and Figure 2.2. Primers were obtained from Eurofins (Germany).

Table 2.1 Primers used in this study

Primer	Sequence (5'-3')	Observation
L2 Express F	aag ttc tgt ttc agg gcc cgG CGG GCA AGG CCA C	pOPINF-L2
L2 Express R	atg gtc tag aaa gct tta TCC GAT CAA CCG GTC GGC	pOPINF-L2
mpL_F1	ACCAGATCCAGGTACCGCC	
mpL_R1	GTGGCCGACTGATCGTCAA	
mpL_F2	CCAGTGCACATCGCCCATTG	
mpL_R2	TCTCACATCCCGTGTAGGACT	
rplA_F	GCGAAGGAACCGGATCTGA	
rplA_R	CGCCTGCGGTCTTTGAC	
rplAfull_NotI_F	AAAGCGGCCGCATCCAGCTGTAGAGTCGAGC	+*NotI*
rplAfull_Not_I_R	AAAGCGGCCGCCTGCGGTCTTTGACGGCTAC	+*NotI*
ampR (+)	ATGTTGCACCCTACCCTG	
ampR (-)	TGGTCTGCGATACCTACT	
Smlt_0009_F	AAAGAATTCAGTAGGAATAACGCCTGAATGC	+*EcoRI*
Smlt_0009_R	AAAGAATTCTGACGCTTACCTTTGTTGTGTG	+*EcoRI*
Smlt_0622_NotI_F	AAAGCGGCCGCCAACGAGCGGGATGTTAGGT	+*NotI*
Smlt _0622_NotI _R	AAAGCGGCCGCCGTCGAAGTGGGCAACAAC	+*NotI*
Smlt2644_F	AAAGAATTCTTGGAGCCACTGTGGAGATTG	+*EcoRI*
Smlt2644_R	AAAGAATTCGGTGGGTCGGGGGTAGAGT	+*EcoRI*
Smlt1636_F	AAAGAATTCGAGGATGGCTGCGCATGAT	+*EcoRI*
Smlt1636_R	AAAGAATTCCAGCAACATCGGCATCTGGA	+*EcoRI*
M13F	GTAAAACGACGGCCAGT	
M13R	AACAGCTATGACCATG	
* SmeZQ-F	TGTCCAGCGTCAAGCACC	
*SmeZQ-R	GCCGACCAGCATCAGGAAG	
*rDNA-F	GACCTTGCGCGATTGAATG	++*rrnB*
* rDNA-R	CGGATCGTCGCCTTGGT	++*rrnB*

*Primers used for RT-qPCR

+Restriction site included in the primer sequence

++Ribosomal gene in *S. maltophilia*

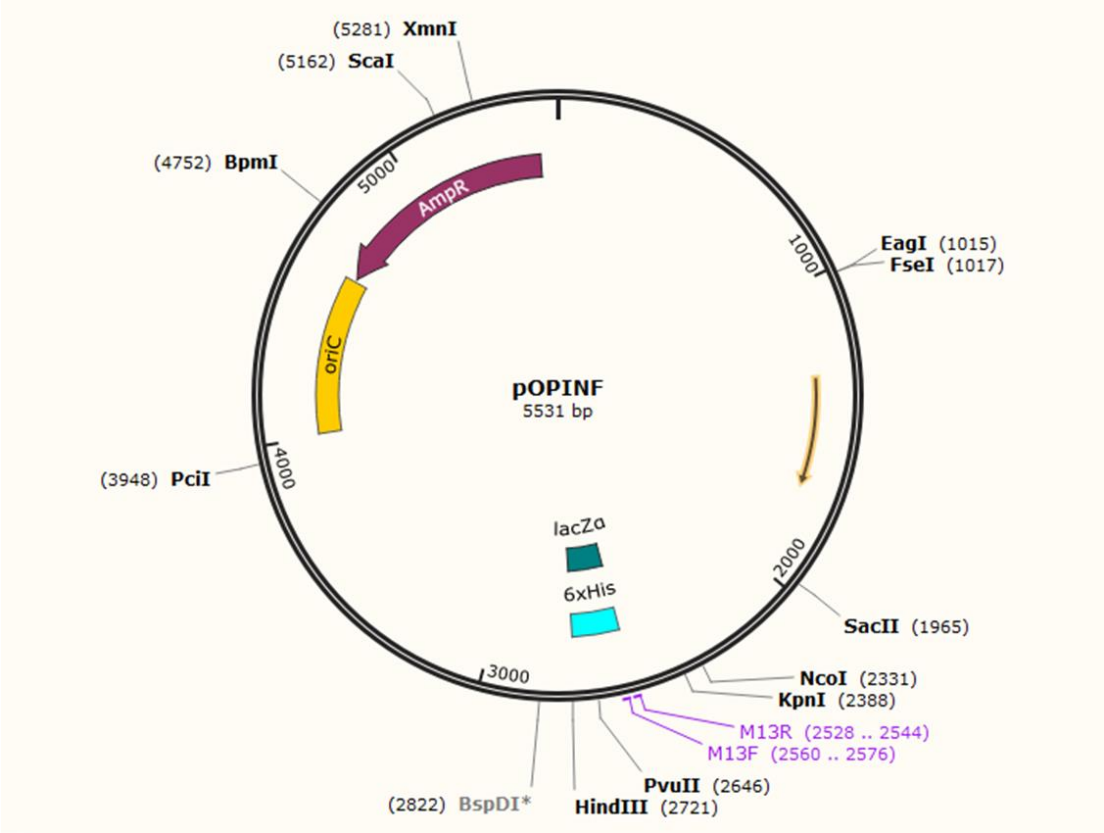


Figure 2.1 Map of pOPINF vector

pOPIN contains an ampicillin selection marker AmpR, two important restriction sites KpnI and HindIII to linearize the vector since the 6X His (cyan) has been strategically located to tag the recombinant protein. LacZα (green) allows blue-white selection screening

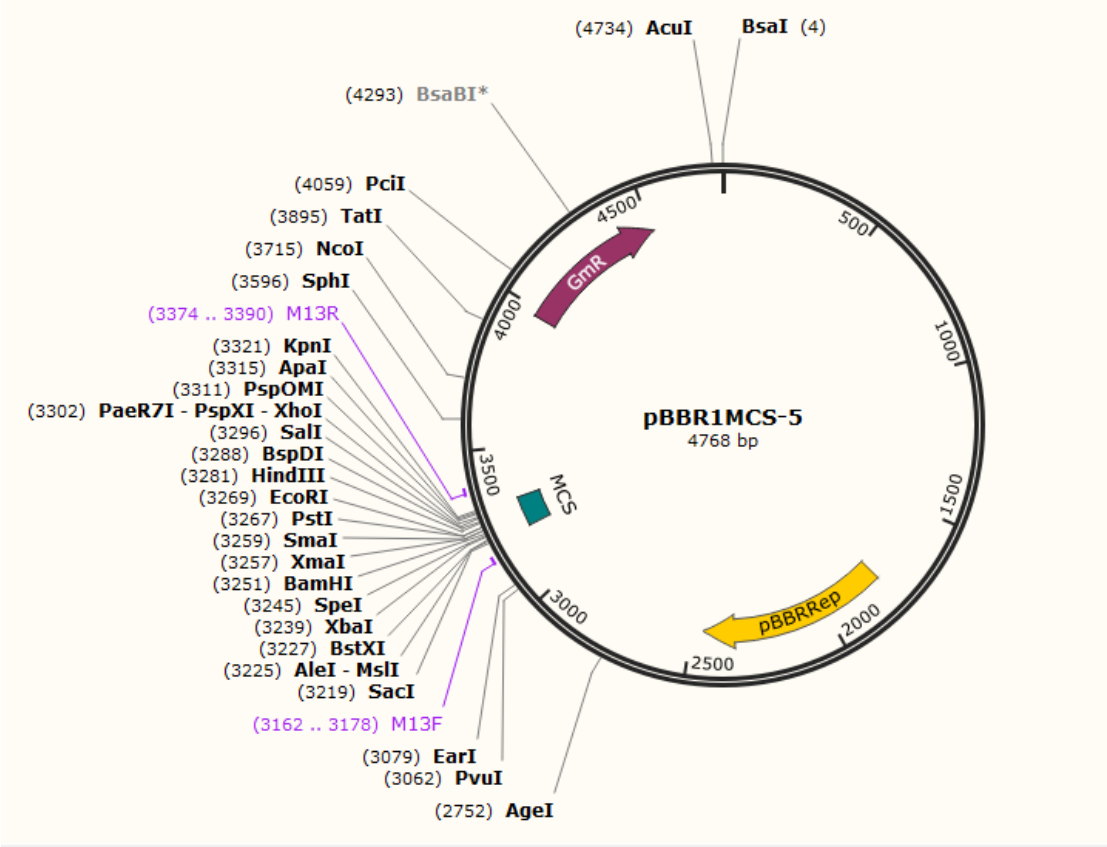


Figure 2.2 Map of pBBR1MCS-5

pBBR1MCS-5 is just one of a series of vectors designed for multiple hosts. pBBR1MCS-5 confers resistance to gentamicin. Multiple Cloning site (green) starts with KpnI and ends with SacI. Insertion of DNA can be confirmed using universals M13 primers. pBBR1MCS-1 confers resistance to chloramphenicol, pBBR1MCS-4 confers resistance to ampicillin.

2.3 Selection of resistant mutants

K279a mutants were selected after exposure to ceftazidime discs on MHA by picking the colonies within the zone of inhibition after using a bacterial suspension that was 100-fold higher than the recommended value according to the CLSI guidelines(194). Lactivicin resistant mutants, minocycline resistant mutants, and levofloxacin resistant mutants were selected by plating 100 µL of an overnight culture grown in NB on MHA containing different concentrations of test antimicrobial (s).

2.4 Siderophore Detection

100 µL of an overnight culture was used to set up a fresh subculture in 10 mL of MHB which was then incubated until the OD_{600} reached 0.5. Cells were centrifuged (4,000 x g, 10 min) and the resulting pellet was resuspended in 10 mL of PBS and centrifuged again (4,000 x g, 10 min). The supernatant was discarded and the pellet was again resuspended in fresh PBS (10mL) and centrifuged (4,000 x g, 10 min). Washed bacterial pellet was then diluted in PBS to prepare a bacterial suspension of OD_{600} 0.2. Ten microliters of the bacterial suspension were spotted on Chrome Azurol S (CAS) agar. CAS agar was made up mixing up 90 mL of MHA and 10 mL of freshly made CAS solution. 100 mL of the CAS solution was made up based on the following description: 60.5 mg of CAS in 50 mL of water, 72.9 mg of hexadecyltrimethyl ammonium bromide HDTMA in 40 mL of water, and 10 mL of 1 mM $FeCl_3$, 10 mM HCl) (195). CAS agar control included 100 µM $FeCl_3$ where no colour change was expected.

2.5 Determining minimal inhibitory concentrations (MICs) of antimicrobials and disc susceptibility testing

The Clinical and Laboratory Standard Institute (CLSI) protocol was followed for disc-test susceptibility testing (196). The clearing zone was measured after 20 h of incubation and values reported as susceptible or resistant according to the CLSI published breakpoints (197). Reduced susceptibility is reported in comparison to a reference strain (K279a) when the difference between clearing zones strain was ≥ 5 mm.

MICs were determined using CLSI broth microtitre assays (194) and interpreted using published breakpoint (197). Briefly, a PBS bacterial suspension was prepared to obtain a stock of OD_{600}=0.01. Antibiotic stocks were prepared considering the breakpoint value for resistance according to the CLSI guidelines and then diluted 1:1000 to use it as the starting concentration from where the two-fold serial dilutions proceed. Final volume in the 96-well cell culture plates (Corning Costar) was 200 µL, obtained by combination of 180 µL of the antibiotic solution and 20 µL of the bacterial suspension. Bacterial growth was determined after 20 h of incubation by measuring OD_{600} values using a POLARstar Omega spectrophotometer (BMG Labtech). The well, corresponding to the antibiotic concentration, with an OD_{600} equivalent to a baseline reading was considered as the final MIC value.

2.6 β-lactamase production/induction assays

To measure the amount of β-lactamase produced by bacteria in the absence or presence of different potential inducing agents, 100 µL of an overnight culture of bacteria was diluted in 10 mL of NB and grown at 37°C until OD_{600} was 0.4. Prospective inducer (s) were then added, or not, as necessary and incubation continued for 2 h before cell extracts were prepared.

2.6.1 Preparation of cell extracts

Cells from a 10 mL culture were pelleted by centrifugation (4,000 x g, 10 min) and pellets resuspended in 100 µL of BugBuster (Ambion. Pellets were transferred to 1.5mL microtube (Eppendorf) before rocking at 70 rpm for 30 min at room temperature. Cell debris and unlysed cells were pelleted by centrifugation (13,000 x g, 5 min) and the supernatant retained as a source of crude cell protein. Protein cell extracts obtained from using the sonication and concentration method described in **2.11** were also used for some experiments. Protein concentrations in cell extracts were determined using the BioRad protein assay dye reagent concentrate. Briefly, 10 µL of cell extract was combined with 90 µL of the dye reagent and volume was made up to 1 mL with water. OD_{599} was measured and Equation 1 used to calculate the protein concentration. The conversion factor was obtained based on a calibration curve using fixed concentrations of BSA.

$$x = \frac{OD_{599} - 0.0392}{0.6603}$$

Equation 1

where:

$$OD_{599} = optical\ density\ of\ the\ protein\ sample$$

$$x = [ng.\mu L^{-1}]$$

2.7 β-lactamase activity assay

β-lactamase activity in crude cell extracts was determined using a POLARstar Omega plate spectrophotometer (BMG Labtech). A meropenem (100 µM) or nitrocefin (40 µM) solution was used as a substrate, each prepared in filtered (0.2 µm) assay buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$ pH 7.0, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM

$MgSO_4 \cdot 7H2O$, 100 µM $ZnCl_2$). Meropenem hydrolysis assay were performed using half-area 96-well UV-translucent plates (Greiner UV-Star. Bio-one), 90 µL of 100 µM meropenem was combined with 10 µL of the cell extract. Nitrocefin hydrolysis assays were performed in Corning Costar 96-well flat bottomed cell culture plates with a combination of 1 µL of cell extract and 179 µL of nitrocefin solution. For meropenem, substrate depletion was followed at 300 nm for 10 min whilst for nitrocefin hydrolysis, product accumulation was measured at 482 nm for 5 min. Final β-lactamase activity was calculated using Equation 2.

$$\frac{nmoles\ of\ substrate}{min.\mu g\ protein} = \frac{\frac{A_*}{e * l} * 1000000 * v}{x * v_{po}}$$

Equation 2

Where:

$$A_* = change\ in\ absorbance\ per\ min$$

Calculated as the linear phase of the reaction in Omega Data Analysis

$$e = extinction\ coefficient\ (M^{-1}.cm^{-1})$$

9600 $M^{-1}cm^{-1}$ for meropenem, 17400 $M^{-1}cm^{-1}$ for nitrocefin

$$l = pathlength\ (cm)$$

Pathlength for liquid in a well in a 96-well plate = 0.56 cm for nitrocefin and 0.62cm for meropenem

$$1000000$$

Conversion to obtain activity in $nmol.mL^{-1}$

$$v = volume\ of\ reaction\ (mL)$$

Volume of reaction was 0.1 (meropenem) or 0.18 (nitrocefin)

$$x = protein\ concentration\ (ng.\mu L^{-1})$$

$$v_{po} = volumen\ of\ protein\ used\ in\ the\ assay\ (\mu L)$$

2.8 Steady-state kinetics and inhibitory concentration (IC_{50}) measurement

Recombinant L1 and L2 proteins were produced in *E. coli* and purified as described in (198) and (**2.9**). Steady state kinetics were determined by assaying enzyme activity using a POLARstar Omega (BMG Labtech) fluorescent plate reader using L1 assay buffer (50 mM HEPES pH 7.5, 10 µg/mL BSA, 10 µM $ZnSO_4$ and 0.01% v/v Triton X-100) or L2 assay buffer (50 mM Tris pH 7.5, 10 µg/mL BSA and 0.01% v/v Triton X-100). Reactions were carried out as described in (192). Reactions were set up in black flat-bottomed 96-well plates (Greiner Bio-one, Stonehouse, UK) with a final volume of 100µL. Final reaction contained 50 pM enzyme and a series of two-fold serial dilutions of the fluorogenic substrate FC5, enabling to measure product accumulation at eight different concentrations at the excitation wavelength 380nm and emission wavelength at 460 nm. A similar process was used for chromogenic substrates meropenem, ceftazidime and aztreonam, but for these, spectrophotometric assay of substrate depletion was measured at 300 nm, 260 nm, 318 nm, respectively in half-area 96 well UV-translucent plates (Greiner UV-Star. Bio-one).

To determine IC_{50}s of inhibitors against L1 and L2, assays were performed in black flat-bottomed 384-well plates with a final volume of 25 µL with enzyme concentration 50 pM, FC5 substrate concentration 5 µM, inhibitor concentration ranged from 1 nM-2.5 mM. Volumes of buffer, enzyme and substrate were dispensed using Multidrop Combi Reagent Dispenser (Thermo Scientific) whereas tested inhibitors were dispensed with Cybi-well (CyBio). Steady state kinetic data were analysed by curve fitting to the Michaelis-Menten equation using Prism software while IC_{50}s curves corresponding to different inhibitor concentrations were logarithm-transformed and fitted by nonlinear regression to equation log(inhibitor) vs. response –(three parameters) where Hill slope= -1.0, using Prism software **Equation 3**.

$$y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{(x - logIC_{50})})}$$

Equation 3

Where:

$$x = log\ of\ inhbitor\ concentration$$

$$y = response, decreasing\ as\ x\ increases$$

$$Top\ and\ Bottom: plateus\ in\ same\ units\ as\ y$$

$$logIC_{50} = same\ units\ as\ x$$

2.9 Expression and purification of L2

For L2 protein expression the putative signal sequence (residues 1-27) was removed. Codons 82-912 of the L2 gene from *S. maltophilia* K279a genomic DNA were amplified with forward and reverse primers L2 express (**Table 2.1**). Primer sequences included extensions which allowed recombination into the pOPINF vector (199), resulting in an L2 construct containing an N-terminal His6 tag, with the tag cleavable with 3C protease. The resultant plasmid was designated pOPINF-L2Δ27.

For protein overproduction, *E. coli* SoluBL21 (DE3) cells (Genlantis) bearing pOPINF-L2Δ27 were grown in six 2 L flasks with 500 mL 2xTY medium containing ampicillin (50µg/mL) and 5 mL overnight culture was added into each flask and grown at 37°C until reaching an OD_{600}=0.9. Protein expression was induced with 1 mM IPTG at 18°C for 16 h. All subsequent purification steps were at 4°C. Cells were harvested by centrifugation (6500 × g, 10 mins) of the 3 L-culture and resuspended in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine TCEP) supplemented with EDTA-free protease inhibitor (Roche). Cells were lysed by two 30,000 psi passages through a cell disruptor. After centrifugation at 100,000 × g for 45 min, the supernatant was incubated for 1.5 h with Ni-NTA resin (Qiagen). Resin was washed with buffer A (50 mM Tris pH 7.5, 400 mM NaCl, 1 mM TCEP) plus 10 mM imidazole, then buffer A plus 0.1% Triton X-100, then buffer A plus 20 mM imidazole. Protein was eluted with 50mM Tris pH 7.5, 200 mM NaCl, 400 mM imidazole, 1 mM TCEP. Imidazole and NaCl concentrations were reduced to 5 mM and 150 mM, respectively, in an Amicon 10 kDa molecular weight cut-off (MWCO) concentrator and His tag removed by overnight incubation with his tagged 3C protease. The digestion mixture was then incubated with Ni NTA resin for 30 min and the flowthrough containing

purified L2 was collected and concentrated to 30 mg/mL in an Amicon 10 kDa MWCO concentrator.

2.10 Fluorescent Hoescht (H) 33342 dye accumulation assay

Envelope permeability in living bacteria was tested using a standard dye accumulation assay protocol (200) where the dye only fluoresces if it crosses the entire envelope and interacts with DNA. Overnight cultures (in NB) at 37°C were used to prepare NB subcultures at 37°C to 0.6 OD_{600}. Cells were pelleted by centrifugation (4000 rpm, 10 min) (ALC, PK121R) and resuspended in 500µL of PBS. The optical densities of all suspensions were adjusted to 0.1 OD_{600}. Aliquots of 180 µL of cell suspension were transferred to a black flat-bottomed 96-well plate (Greiner Bio-one, Stonehouse, UK). Eight technical replicates, for each strain tested, were in each column of the plate. The plate was transferred to a POLARstar spectrophotometer (BMG Labtech) and incubated at 37°C. Hoescht dye (H33342, 250 µM in water) was added to bacterial suspension of the plate using the plate-reader's auto-injector to give a final concentration of 25 µM per well. Excitation and emission filters were set at 355 nm and 460 nm respectively. Readings were taken in intervals (cycles) separated by 150 s. 31 cycles were run in total. A gain multiplier of 1300 was used. Results were expressed as absolute values of fluorescence versus time.

2.11 Proteomics

500 µL of an overnight culture were transferred to 50 mL NB and cells were grown at 37˚C to 0.6 OD_{600}. Cells were pelleted by centrifugation (10 min, 4,000 ×g, 4°C) and resuspended in 20 mL of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1 s on, 0.5 s off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4°C to pellet intact cells and large cell debris; the supernatant was removed and concentrated (Amicon 3 kDa cutoff filter) for analysis of total cell protein. Alternatively, for envelope preparations, the supernatant was not concentrated, and instead, subjected to centrifugation at 20,000 rpm for 60 min at 4°C using the above rotor to pellet total envelopes. To isolate total envelope proteins, this total envelope pellet was solubilised using 200 µL of 30 mM Tris-HCl pH 8 containing 0.5% (w/v) SDS.

Protein concentrations in all samples were quantified using Biorad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions. Protein concentration was calculated using Equation 1. Proteins (2.5 µg/lane for total cell proteomics or 5 µg/lane for envelope protein analysis) were separated by SDS-PAGE (see below) using 11% acrylamide, 0.5% bis-acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. Gels were resolved at 200 V until the dye front had moved approximately 1 cm into the separating gel. Proteins in all gels were stained with Instant Blue (Expedeon) for 20 min and de-stained in water.

The 1 cm of gel lane was subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd). The resulting peptides from each gel fragment were fractionated separately using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a 250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min, 6-15% B over 58 min, 15-32% B over 58 min, 32-40% B over 5 min, 40-90% B over 1 min, held at 90% B for 6 min and then reduced to 1% B over 1 min) with a flow rate of 300 nL/min. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization MS at 2.1 kV using a stainless-steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyze the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalized collision energy, 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt *S. maltophilia* strain K279a database (4365 protein entries; UniProt accession UP000008840) using the SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set

at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5 %. The Proteome Discoverer software generates a reverse "decoy" database from the same protein database used for the analysis and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identifications. The minimum cross-correlation factor filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5 % based on the number of random false positive matches from the reverse decoy database. Thus, each data set has its own passing parameters. Protein abundance measurements were calculated from peptide peak areas using the Top 3 method (201) and proteins with fewer than three peptides identified were excluded. The proteomic analysis was repeated three times for each parent and mutant strain, each using a separate batch of cells. Data analysis was as follows: all raw protein abundance data were uploaded into Microsoft Excel. Raw data from each sample were normalised by division by the average abundance of all 30S and 50S ribosomal protein in that sample. A one-tailed, unpaired T-Test was used to calculate the significance of any difference in normalised protein abundance data in the three sets of data from the parent strains versus the three sets of data from the mutant derivative. A *p*-value of <0.05 was considered significant. The fold change in abundance for each protein in the mutant compared to its parent was calculated using the averages of normalised protein abundance data for the three biological replicates for each strain.

2.12 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were prepared according to amount of protein required for proteomics analysis and then mixed with SDS-PAGE loading buffer (100mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol) and heated at 95°C for 5 min. The separating gel was obtained from combining 4.2 mL of 30% acrylamide/bisacrylamide solution (37.5:1 Bio-Rad), 2.5 mL of separating buffer (1.5M Tris-Base pH 8.8, 4% SDS), 2.3 mL of Elgastat water, 100µL of 10% w/v ammonium persulphate, and 20 µL of 10% TEMED. The stacking gel solution was made up of 2 mL of 30% acrylamide/bisacrylamide solution (37.5:1, Bio-Rad), 2.5 mL of the stacking buffer (0.5M Tris-Base pH 6.8, 0.4% SDS), 4.5mL of water, 100 µL of 10% w/v

ammonium persulphate, and 20 µL of 10% TEMED. High- molecular- weight pre-stained protein marker was used (SeeBlue Plus2, Life Technologies). Gels were run at 170 V until they reached the desired distance for proteomics analysis. InstantBlue stain (Expedeon) was used for 15 min to visualize the proteins and then removed with Elgastat water.

2.13 Polymerase Chain Reaction and Sequence Analysis

A bacterial colony was resuspended in 100 µL of molecular biology grade water and heated at 100˚C for 5 min. Sample was centrifuged at 13000 rpm for 5 min. One microlitre of the resulting supernatant was used as a DNA template for downstream PCR reactions. Alternatively, a single colony was resuspended directly in the PCR reaction and used as a template.

2.13.1 Polymerase chain reaction (PCR)

When using MyTaq PCR Master Mix (BioLine), PCR reactions were set up in 0.2 mL thin-walled PCR tubes using 12.5 µL of MyTaq PCR Master Mix, 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 10.5 µL of molecular biology grade water and 1 µL of DNA template. A PCRmax Alpha Cycler 2 Thermal Cycler (Cole-Parmer) was used to carry out the reaction. The cycling conditions were the following: 1 cycle of 95˚C for 1 min, 30 cycles of : 95˚C for 15 s, 55˚C-68˚C for 15 s, and 72˚C for 10 s, 1 cycle of 72˚C for 10 min for final extension. When using Phusion ® High-Fidelity DNA Polymerase (New England Biolabs) for high-GC-content DNA fragments, PCR reaction was set up using 5µL of 5X Phusion GC Buffer, 0.5µL of dNTPs (10mM), 1.25 µL of forward primer (10µM), 1.25 µL of reverse primer (10µM), 0.75µL of DMSO, 0.25 µL of Phusion DNA Polymerase, 1µL of DNA template, and 15 µL of molecular biology grade water. The cycling conditions were the following: 1 cycle of 98˚C for 30 s, 30 cycles of: 98˚C for 10 s, 60˚C-68˚C for 30 s, and 72˚C for 30 s, 1 cycle of 72˚C for 10 min for final extension.

PCR products were visualized in 1% Tris/Acetate/EDTA agarose gel. One gram of agarose was dissolved in 100 mL TAE buffer (0.04 M Tris Base, 0.02 M Glacial Acetic Acid, 1 mM EDTA pH 8.0) with ethidium bromide (0.1 $\mu g.mL^{-1}$). 10 µL PCR products obtained with Phusion DNA Polymerase required being mixed with 2 µL of 5X DNA loading buffer whilst PCR products obtained with MyTaq PCR Master Mix include loading buffer in the PCR reaction. 3 µL of Hyperladder (1 Kb, BioLine) was used as a marker. Samples were run at 120 V for 30 min and then agarose gels visualised using the GeneGenius Gel Imaging System (Syngene).

PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. DNA concentration of purified samples was quantified using NanoDrop Lite spectrophotomer (Thermo Scientific). Samples were sent to Eurofins (Germany) according to the sample submission guide provided by Eurofins. Sequences obtained were analysed with ClustalW OMEGA or MultiAlignPro. Alignments were represented using ESPript 3.0.

2.14 Reverse Transcription Quantitative PCR (RT-qPCR)

2.14.1 RNA Isolation and DNase Treatment

From an overnight culture, 200 µL were used to set up a 20 mL NB subculture. Cells were grown to OD_{600} 0.6. 10 mL of the culture was centrifuged at 4, 000 × g for 10 min and then resuspended in 2 mL of NB. Four mililitres of Bacterial RNA Protect Reagent (Qiagen) was added to the sample. The stabilized culture was centrifuged for 10 min at 4, 000 × g and the supernatant discarded.

Total RNA was isolated from the cell pellet using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. However, a modification was added after incubation of the sample with lysozyme (15 $mg.mL^{-1}$, TE buffer) at 37˚C for 10 min, and immediate addition of buffer RLT. Samples were transferred to a lysing tube, containing acid-washed 0.1 mm silica spheres, (Lysing matrix B, MP Biochemicals, Eschwege, Germany). Next, lysing tubes were processed in a Hybaid Ribolyser (Hybaid International, UK) for two cycles of 45 s at speed 6. Samples were then centrifuged at 13 000 rpm at 4˚C for 1 min. The resulting supernatant was used to continue with RNA isolation using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Purified total RNA was quantified using Nanodrop Lite (Thermo Scientific) and stored at -70 ˚C until required.

DNA was removed from RNA samples using Turbo DNase (Life technologies) according to the manufacturer's instructions.

2.14.2 RT-PCR

RNA concentrations in DNase treated RNA samples were quantified using Nanodrop Lite (Thermo Scientific) and then standardized to 100 $ng.µL^{-1}$. One microgram of RNA from each sample was converted to cDNA using qScript cDNA SuperMix (Quantabio). cDNA was synthesized with the following conditions: 25˚C for 5 min, 42˚C for 30 min, 85˚C for 5 min. All these steps were carried out without in a

PCRmax Alpha Cycler 2 Thermal Cycler (Cole-Parmer) without an initial heating of the lid of. cDNA was then stored at -20˚C until required or most of the time, it was used immediately.

2.14.3 qPCR

Real-time quantitative PCR (qPCR) reactions were prepared using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). Briefly, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 7µL molecular biology grade water, 1 µL of cDNA and 10 µL of PowerUp™ SYBR™ Green Master Mix (2X) were mixed to set up a 20 µL reaction. qPCR was carried out in a StepOnePlus ™ Real-Time PCR System (Applied Biosystems). Each qPCR analysis had three biological replicates (i.e. cDNA from three separate preparations of RNA) and 4 technical replicates (assays using a given a preparation of cDNA). The cycling conditions were the following: 1 cycle of 95˚C for 2 min, 40 cycles of: 95˚C for 2 s, 60˚C for 30 s. Relative quantification was calculated using the comparative method (202).

2.15 Whole genome sequencing to Identify Mutations

Whole genome resequencing was performed by MicrobesNG (Birmingham, UK) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic (203) and assembled into contigs using SPAdes 3.10.1 (http://cab.spbu.ru/software/spades/). Assembled contigs were mapped to reference genome for *S. maltophilia* K279a (173) obtained from GenBank (accession number NC_010943) using progressive Mauve alignment software (204).

2.16 DNA manipulation

2.16.1 Plasmid isolation, digestion and purification

Plasmid preparations were obtained from overnight cultures (10 mL) using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Plasmid yield was measured using a NanoDrop Lite spectrophotometer (Thermo Scientific).

Restriction digestions were carried out according to the manufacturer's instructions. Three micrograms of plasmid DNA were digested with 3 units of the appropriate restriction enzyme (New England Biolabs), 6 µL of the restriction buffer (10X), when required, 3 µL of Shrimp Alkaline Phosphatase (New England Biolabs),

and molecular biology grade water to complete a final volume reaction of 60 µL. The reaction was incubated at 37˚C for 1- 3 h depending on the specificity of the enzyme (high-fidelity or standard) and then visualized and purified from a 1% w/v agarose gel (see above). Bands were cut out with a scalpel and DNA purified using a Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.16.2 DNA ligation

Ligations reactions were carried out by adding a 3:1 molar ratio of insert to the vector, 1 unit of T4 DNA ligase (Promega), 1 µL of ligase buffer (10X) and molecular biology grade water to complete a final volume reaction of 10 µL. Ligation reaction was incubated overnight at 4˚C. When LigaFast ™ Rapid DNA Ligation System (Promega) ligations were performed, the same 3:1 molar ratio of insert to the vector and 1 unit of T4 DNA ligase (Promega) was used but 5 µL of rapid ligation buffer (2X) and molecular biology grade water to complete a final volume reaction of 10 µL. Rapid Ligation reactions were incubated at room temperature for 30 min.

2.17 Transformation

2.17.1 Making chemically competent cells and heat-shock

500 µL of an overnight culture was used to set up a 50 mL fresh subculture in LB broth at 37°C until the OD_{600} 0.6. The culture was then aliquoted in 25 mL falcon tubes and centrifuged at 4,000 × g for 10 min. Cells were then resuspended in 25 mL of sterile ice cold $CaCl_2$ (100 mM) and placed on ice for 30 min. Cells were centrifuged at 4,000 × g for 10 min and supernatant was removed. The resulting cell pellet was resuspended in 1 mL of sterile ice cold CaCl2 (100 mM, 15% v/v Glycerol). Cells were stored as 75 µL aliquots at -70˚C.

Transformation of chemically competent cells was carried out by heat shock. 75 µL of the chemically competent cells were incubated with the 150 ng of the plasmid or 5 µL of the ligation reaction for 30 min, heat-shocked at 42˚C for 1 min and incubated on ice for 3 min before adding 900 µL of pre-warmed SOC medium. Heat-shocked cells were then incubated in a shaking incubator at 37°C for 1 h. Cells were centrifuged at 8000 rpm for 2 min and then resuspended in 100 µL of the same SOC medium. 100 µL was then plated on 1.6% LB agar (containing 20 $\mu g.mL^{-1}$ chloramphenicol, 15 $\mu g.mL^{-1}$ gentamicin or 100 $\mu g.mL^{-1}$ ampicillin, depending upon the plasmid being used). When making blue/white selection screening, apart from the

antibiotic selection marker, plates also contained isopropyl-β-D-1-thiogalactopyranoside (IPTG 0.1 mM) and X-Gal (40 $\mu g.mL^{-1}$). Colonies were selected for colony PCR from where PCR products were sequenced and analysed to confirm the presence of the insert in the vector.

2.17.2 Making electro-competent cells and Electroporation

One mililitre of an overnight culture was used to set up a 100 mL fresh subculture in LB broth grown at 37°C until the OD_{600} 0.6. The culture was then aliquoted in 50 mL falcon tubes and centrifuged at 4,000 × g for 10 min. The supernatant was discarded and cells were washed in 50 mL of sterile ice cold water to be then pelleted at 4,000 × g for 10 min. A second washing step followed, and cells were resuspended in sterile ice cold 12.5% v/v glycerol and then pelleted at 4,000 × g for 10 min. A second washing step with sterile ice cold 12.5% glycerol and a second centrifugation at 4,000 × g for 10 min followed. Finally, 300 µL of sterile ice cold 12.5% glycerol was used to resuspend the final cell pellet. Cells were stored as 75 µL aliquots at -70°C.

Electroporation was carried out by incubation of 75µL of the electro-competent cells with 150 ng of DNA for 5 min in ice. The mixture was then transferred to an ice cold 2 mm-electroporation cuvette (Cell Project). A pulse was applied with a Bio-Rad Gene Pulser (Bio-Rad, UK) at 2.5 kV, 25 µF and 200 Ω. 950 µL of pre-warmed LB broth was added. The cuvette containing cells, DNA and broth was incubated at 37°C for 1 h. Cells were transferred to a 1.5 mL microtube (Eppendorf) and centrifuged at 8000 rpm for 2 min and then resuspended in 100 µL of the same LB medium and the entire sample was plated on 1.6% LB agar (containing antibiotic selection as described above). Colonies were selected for colony PCR from where PCR products were sequenced and analysed to confirm the presence of the insert in the vector.

2.17.3 L2 Crystallisation, Data Collection and Structure Modelling

Initial L2 crystals grew using sitting-drop vapour diffusion in 96-well MRC 2-drop plates (Molecular Dimensions) with the Morpheus sparse matrix screen (205). Conditions were refined in CrysChem 24-well sitting-drop plates (Hampton Research, 18°C), and diffraction-quality crystals were obtained by mixing 1 µL of L2 protein (42 mg/mL) with 1.5 µL reagent (10% w/v PEG 20000, 20% v/v PEG MME 550, 0.02 M DL-Glutamic acid; 0.02 M DL-Alanine; 0.02 M Glycine; 0.02 M DL-Lysine; 0.02 M DL-Serine, 0.1 M bicine/Trizma base pH 8.5) and equilibrated against 500 µL reagent. L2

complexes were obtained by soaking crystals in bicyclic boronate 2 (5 min, 2.5 mM) or avibactam (40 min, 5 mM) dissolved in reservoir reagent. L2 crystals were cryoprotected using reservoir solution plus 20% glycerol and flash frozen in liquid nitrogen. Crystallographic data were collected at 100K (I04-1, I04 or I03, Diamond Light Source, UK) and integrated in XDS(206) or DIALS (207), and scaled in Aimless in the CCP4 suite (208). Phases were calculated by molecular replacement in Phaser (209) using PDB 1O7E (unpublished) as a starting model. Avibactam and boronate structures, covalently bound to Ser70, and geometric restraints were generated using Phenix eLBOW (210). Structures were completed by iterative rounds of manual model building in Coot (211)and refinement in Phenix (212). Structure validation was assisted by Molprobity (213) and Phenix (212). Figures were prepared using Pymol (Schrodinger).

3 Characterisation of new mechanisms of ceftazidime resistance in *S. maltophilia*

3.1 Introduction

S. maltophilia clinical isolates are resistant to almost all β-lactams because of the production of two β-lactamases: L1, a subclass B3 metallo-β-lactamase and L2, a class A ESBL (214) Production of L1 and L2 is co-ordinately controlled by AmpR, a LysR-type transcriptional activator and induced during β-lactam challenge of cells (215). Where previously characterised, AmpR regulators have been shown to bind two ligands in a competitive manner (160, 216). As summarised in **Figure 3.1**, the AmpR activator ligand, an anhydro-muramyl-penta-peptide is produced during β-lactam challenge via the concerted actions of lytic transglycosylases, which release N-acetylglucosamine-anhydro-muramyl-peptides from peptidoglycan (217) and AmpG, a permease that transports them into the cytoplasm (218, 219). NagZ, an enzyme that removes the N-acetylglucosamine moiety is also necessary to release the AmpR activator ligand in some species (220), though not in *S. maltophilia* (221). The AmpR repressor ligand is a UDP-muramyl-penta-peptide (222). It is produced via sequential addition of amino acids to a UDP-muramyl substrate, via four separate ligase enzymes, MurC (223), MurD (224), MurE (225) and MurF (226), with the last adding a D-alanine/D-alanine dipeptide made by Ddl (227). Mpl is an enzyme that can ligate a ready-made penta-peptide onto the UDP-muramyl substrate, skipping the MurC, D, E, Ddl and MurF ligation reactions, each of which requires ATP hydrolysis (228). This Mpl catalysed reaction therefore saves considerable amounts of energy for the cell. Its penta-peptide substrate comes from breakdown of anhydro-muramyl-penta-peptides by the peptide amidase AmpD. In this way, breakdown of the anhydro-muramyl-penta-peptide AmpR activator ligand by AmpD is also directly linked to production of the UDP-muramyl-penta-peptide AmpR repressor ligand by Mpl (215, 217, 229, 230) (**Figure 3.1**).

Despite inducible production of L1 and L2 β-lactamases many *S. maltophilia* clinical isolates remain ceftazidime susceptible (152). However, mutants that have acquired ceftazidime resistance can easily be identified in the laboratory, and ceftazidime resistant isolates are commonly encountered in the clinic. In many cases, these mutants hyperproduce L1 and L2 (214). Mutations that reduce AmpD function are known to boost L1/L2 production, because the AmpR activator ligand is broken down much less if AmpD is damaged (231). Mutations that (presumably) increase

peptidoglycan turnover, releasing more muropeptides, also activate L1/L2 production, e.g. those in PBP1A, encoded by *mcrA* (232) and in the lytic transglycosylase MltD, because this mutation stimulates the net production of lytic transglycosylase activity in the cell (233). Mutations in AmpR also activate L1/L2 production (160). Prior to the start of this PhD project, the group had characterised ceftazidime resistant, β-lactamase hyper-producing laboratory selected mutants derived from the extremely well studied clinical isolate K279a. One of these mutants, KCAZ14, was wild-type for *ampR*, *ampD*, and *mcrA* (214) suggesting the participation of an additional gene in β-lactamase hyperproduction. The first part of this chapter describes identification of this additional regulatory gene,

The same study (214) also identified ceftazidime resistant mutants that did not hyperproduce β-lactamase. It was hypothesised that these mutants might have reduced accumulation of ceftazidime in the periplasm due to reduced porin expression or increased ceftazidime efflux. Accordingly, the second part of this chapter describes identification of this novel mechanism of ceftazidime resistance.

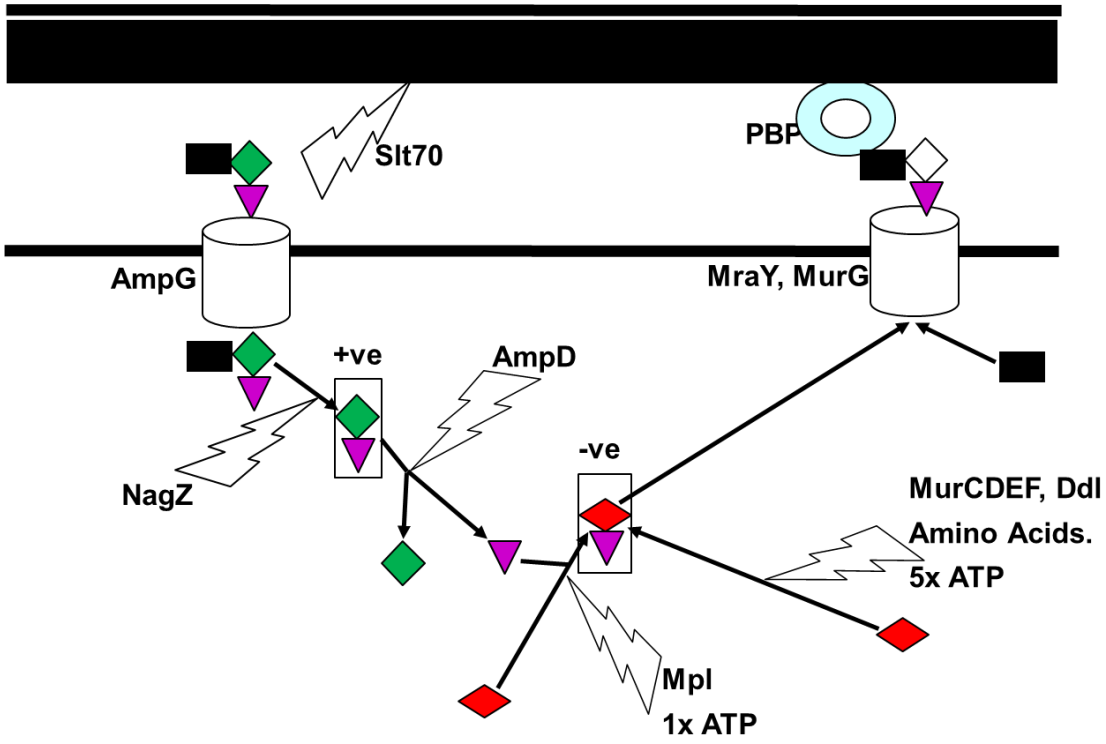


Figure 3.1 Role of Mpl in peptidoglycan recycling and AmpR activation

The schematic shows that N-acetylglucosamine (black square)-anhydro-muramyl (green diamond)-penta-peptide (purple triangle) is removed from peptidoglycan by lytic transglycosylases such as Slt70 and enters the cytoplasm through the permease AmpG. NagZ removes the N-acetylglucosamine group to produce the anhydro-muramyl-penta-peptide AmpR activator ligand ("+ve"). AmpD then releases the penta-peptide ready to be linked to a UDP-muramic acid molecule (red diamond) by Mpl to produce the UDP-muramyl-penta-peptide AmpR repressor ligand ("-ve"). This can then be further incorporated into the biosynthetic pathway and processed by MurG and MraY, which add N-acetylglucosamine and penicillin binding proteins, which add these high energy N-acetylglucosamine-muramyl (white diamond)-penta-peptide substrates to the nascent peptidoglycan strand. UDP-muramyl-penta-peptide formation can also occur without peptidoglycan recycling, through the sequential addition of amino acids to UDP-Muramic acid. However, this requires five moles of ATP per mole of UDP-muramyl-penta-peptide, whilst the recycling pathway only requires one.

3.2 Results and discussion

3.2.1 Involvement of *mpl* mutation in β-lactamase hyperproduction in *S. maltophilia*

To identify the mechanism for β-lactamase hyperproduction in the previously identified ceftazidime resistant mutant KCAZ14 whole genome sequencing was performed. The only mutation identified in KCAZ14 was a deletion of 18 nucleotides in the *mpl* gene, deleting amino acids 141-146 of Mpl (**Figure 3.2**). The level of β-lactamase production was similar for this *mpl* mutant KCAZ14, KCAZ10, a previously identified *ampD* loss of function mutant (214) and KM11, a previously identified *ampR* activatory mutant (160) (**Table 3.1**).

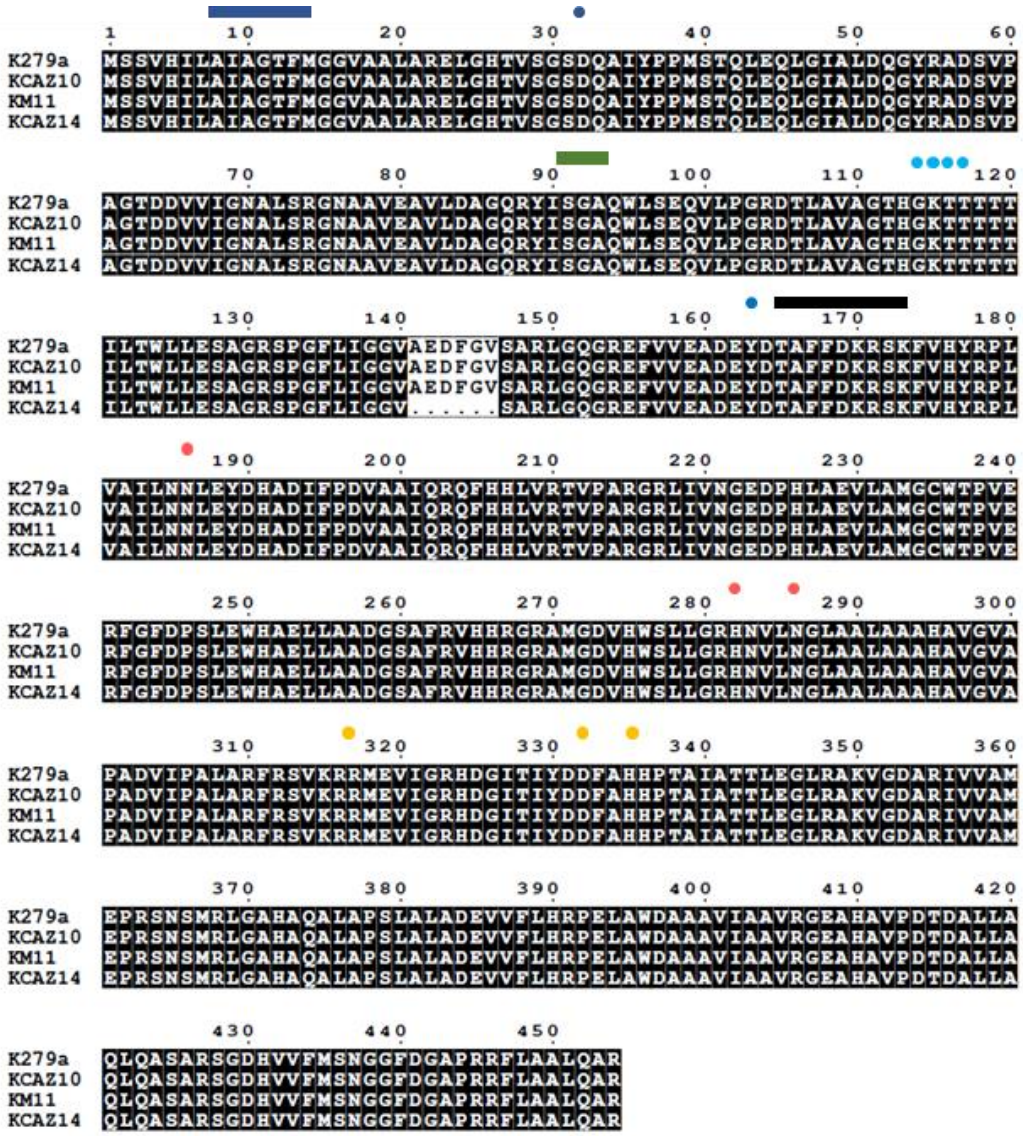


Figure 3.2 Mpl amino acid sequence comparison from K279a and β-lactamase hyperproducing mutants

Alignment of predicted translations of PCR sequences that confirmed the Mpl mutation (non-highlighted region for KCAZ14 residues 141-146) found during whole genome sequencing analysis. Residues 165-176 (black bar) and 91-93 (green bar) may be important for Mpl function given its uniqueness when compared to Mur ligases. Blue bar (residues 8-14) and blue circle (Asp32) are likely involved in UTP binding; blue circle (Tyr163) may participate in substrate recognition. Pink circles (Asn186, His282, His286) are important for ATP especifically adenine binding while orange circle (Asp332) allows ribose binding. Gly114-Thr117 (light blue circles), Arg317, His335 (orange circles) likely involved in tri-phosphate binding. Residues likely involved in metal binding are Thr116, His191, and Glu162 (234). The alignment was performed with CLUSTAL Omega and ESPript 3.

3.2.2 *mpl* complementation in KCAZ14 restores ceftazidime susceptibility

To confirm involvement of *mpl* loss in the β-lactamase hyper-producing, ceftazidime resistant phenotype of KCAZ14, complementation was attempted *in trans*. K279a *mpl* was amplified by PCR as previously (214) with primers mpl_F1 and mpl_R2 (**Table 2.1**) . The product was blunt-end ligated into pBBR1MCS-5 (Gm^R) (235, 236) digested with SmaI and the resulting recombinant plasmid used to transform KCAZ14 to gentamicin resistance (15 $\mu g.mL^{-1}$) via electroporation **Figure 3.3**. The ceftazidime MIC against KCAZ14 (pBBR1MCS-5) was 64 $\mu g.mL^{-1}$ and reduced to 4 $\mu g.mL^{-1}$ in KCAZ14 (pBBR1MCS-5::*mpl*), the same as the MIC against wild-type K279a. Production of β-lactamase was also reduced to wild-type levels in KCAZ14 (pBBR1MCS-5::*mpl*) (**Table 3.1**) and disc susceptibility testing showed an increase in the zone of clearing around a ceftazidime disc for KCAZ14 (pBBR1MCS-5::*mpl*) relative to KCAZ14 (pBBR1MCS-5) adding further confirmation of successful complementation (**Figure 3.4**).

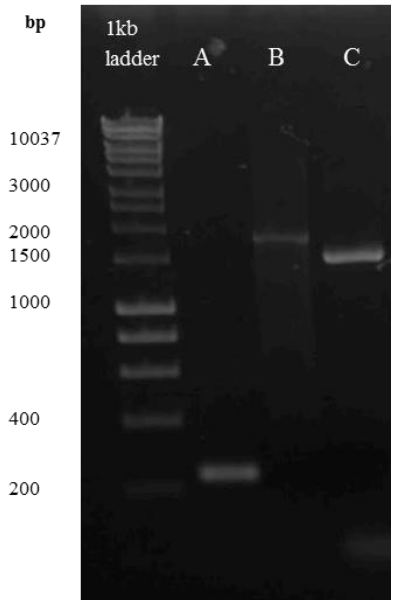


Figure 3.3 *mpl* PCR products obtained during complementation of KCAZ14Δ*mpl*

A) Amplification of the cloning vector insert site flanking region using M13F and M13R primers corresponding to ~250bp (i.e. no insert) from KCAZ14 (Δ*mpl*) carrying pBBR1MCS-5 B) Amplification of the insert site flanking region using M13F and M13R primers corresponding to ~1700bp (the expected size for *mpl* insertion plus some vector sequence) from KCAZ14 (Δ*mpl*) carrying pBBR1MCS-5::*mpl* C) Amplification of *mpl* using primer mpl_F1 and mpl_R2 corresponding to 1546bp K279a (positive control)

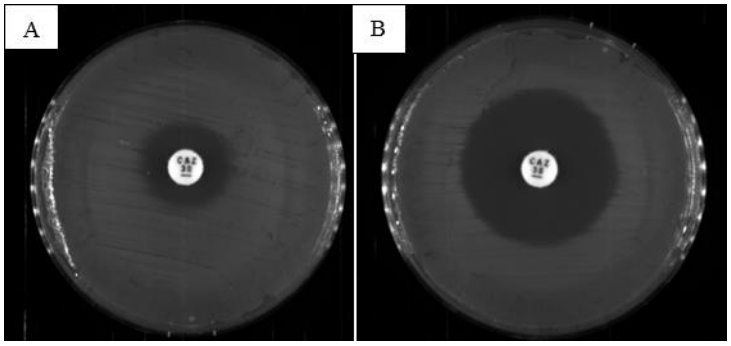


Figure 3.4 Complementation of the KCAZ14 (Δ*mpl*) mutant restores ceftazidime activity

A) KCAZ14 (*Δmpl*) carrying pBBR1MCS-5 ceftazidime disc zone of inhibition= 13 mm **B)** KCAZ14 (*Δmpl*) carrying pBBR1MCS-5::*mpl* ceftazidime disc zone of inhibition= 27 mm

3.2.3 *mpl* mutations are common in *S. maltophilia* clinical isolates

There are four ceftazidime resistant, β-lactamase hyperproducing *S. maltophilia* clinical isolates in the group's collection: isolates 49-6147, 3800 and 98 (214) and ULA-511 (186) (**Table 3.1Error! Reference source not found.**). Isolate 98 has an Insertion Sequence element disrupting *ampD* (214). Using PCR sequencing, a mutation causing an Ala85Gly change in Mpl was also found in isolate 98, but the same mutation is carried by ~5% of *S. maltophilia* genomes in the Genbank database so is probably insignificant. The other three β-lactamase hyperproducing clinical isolates tested do have *mpl* mutations, however. In 49-6147, the mutation causes the deletion of amino acids 92-109, which disrupts the conserved Ser-Gly-Pro region (234). In 3800, there is a frameshift at codon 368 and in ULA-511 there is a nonsense mutation at codon 360.

Table 3.1 β-Lactamase activity (nmol.min^{-1}.µg^{-1} protein nitrocefin hydrolysis activity in cell extracts) observed in *S. maltophilia* K279a and in ceftazidime resistant K279a mutants and clinical isolates carrying different mutations.

Isolate	Mean β-lactamase activity ±SEM	Relevant amino acid changes (Relative to K279a)
K279a	0.02±0.004	WT
KM11	0.99±0.03	Asp135Asn in AmpR
KCAZ10	1.52±0.04	159-168del in AmpD
KCAZ14	0.72±0.01	140-146del in Mpl
49-6147	0.45±0.12	92_109del Mpl
3800	0.73±0.03	Truncation at 368 in Mpl
98	1.76±0.07	IS insertion in *ampD*; Ala85Gly* in Mpl
ULA-511	1.19±0.01	Truncation at 360 in Mpl
KCAZ14 (pBBRMCS-5)	1.14±0.10	
KCAZ14 (pBBRMCS-5::*mpl*)	0.03±0.003	

*Random Genetic Drift

Mutations were identified by PCR sequencing, except for KCAZ14, which was identified by WGS and confirmed by PCR sequencing

WT: Wild type

The result of Mpl loss in KCAZ14 and these clinical isolates will be a build-up of penta-peptides released by AmpD (**Figure 3.1**). Even though there are other enzymes that can break these penta-peptides down (229, 237), it seems reasonable to hypothesise that this net accumulation of penta-peptide will affect AmpD activity by feedback inhibition, increasing the concentration of its substrate, the AmpR activator ligand, causing β-lactamase hyper-production (230). This has not been confirmed however.

This is the first report of *mpl* disruption causing β-lactamase hyperproduction in *S. maltophilia*, and to find it in 3/4 clinical isolates was striking. It is also interesting to find that *mpl* loss of function mutations have been seen to accumulate in *P. aeruginosa*

populations carried by people with Cystic Fibrosis during long term colonisation in two separate studies (238, 239) and also in 3/4 patients with *P. aeruginosa* mediated ventilator associated pneumonia (240). Indeed, *mpl* mutation has been identified as a cause of AmpC β-lactamase hyperproduction in one *P. aeruginosa* PAO1 laboratory selected transposon-insertion mutant (241). Whilst this did not dramatically increase β-lactam MICs (241), PAO1 is relatively permeable to β-lactams, because it lacks many of the efflux pump/porin altering mutations seen in clinical isolates (242). Therefore, it would seem reasonable to propose that these clinically acquired *P. aeruginosa mpl* mutations are being selected by β-lactam therapy. We have a small collection of ceftazidime resistant *P. aeruginosa* clinical isolates, of which 2/5 have previously been confirmed to hyperproduce AmpC (243). Both have a mutation in *mpl*, according to WGS. The mutations in isolates 86-14571 and 73-56826 cause Met297Val and an Arg103His changes in Mpl, respectively. We conclude, therefore, that *mpl* loss in *S. maltophilia* and *P. aeruginosa* is a clinically important and previously under-reported cause of β-lactamase hyperproduction and acquired β-lactam resistance.

3.2.4 Selection and initial characterisation of non-β-lactamase hyperproducing ceftazidime resistant mutants

As previously described, ceftazidime resistant mutants can be selected from K279a that do not hyperproduce β-lactamase (214). To identify the mechanism (s) involved, ceftazidime resistant mutants were selected from K279a as described in section **2.4**. All mutants were tested for elevated basal levels (i.e. measured in the absence of β-lactam challenge) of β-lactamase activity and mutants with basal β-lactamase activity, similar to K279a (~0.02 nmol of nitrocefin hydrolysed.min^{-1}.μg^{-1} of extracted protein) were taken forward for study. Of these, mutants M1 and M52 are exemplars. They are not β-lactamase hyperproducers **Table 3.2** and yet susceptibility to all β-lactams tested was reduced, as shown by an observed reduction in the inhibition zone diameter around various β-lactam discs. However, where non-β-lactams were tested the impact on susceptibility (zone diameter) of the mutations wasminimal (**Figure 3.5**).

Table 3.2 β-Lactamase activity of non- β-lactamase hyperproducing ceftazidime resistant mutants

Isolate	Mean β-lactamase activity ±SEM
K279a	0.02±0.004
M1	0.02±0.002
M52	0.04±0.013

β-lactamase activity (nmol.min^{-1}.µg^{-1} protein nitrocefin hydrolysis activity in cell extracts) calculated as described in section **2.7**

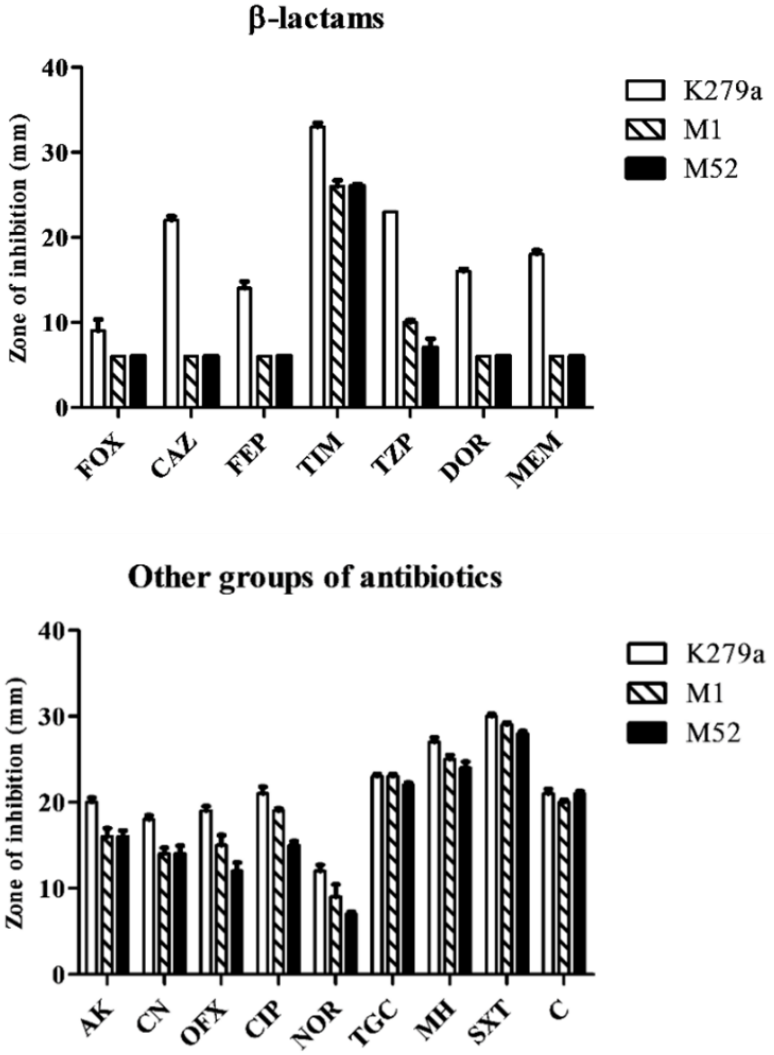


Figure 3.5 Antibiotic susceptibilities for non-β-lactamase hyperproducers

Growth inhibition zone diameters (mm) of ceftazidime resistant mutants (M1 and M52) in comparison with the parental strain (K279a). Smaller zone diameters means reduced susceptibility. Disc susceptibility was performed using MHA and antibiotic discs for **β-lactam group**: cefoxitin (FOX 30 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg), ticarcillin-clavulanic acid (TIM 85 µg), piperacillin-tazobactam (TZP 110 µg), doripenem (DOR 10 µg), meropenem (MEM 10 µg). **Aminoglycosides:** amikacin (AK 30 µg) gentamicin (CN 10 µg). **Quinolones**: ofloxacin (OFX 5 µg), ciprofloxacin (CIP 5 µg), norfloxacin (NOR 10 µg). **Tetracyclines:** tigecycline (TGC 15 µg), Minocycline (MH 30 µg), trimethoprim-sulfamethoxazole (SXT 25 µg). **Chloramphenicol** (C 30 µg). Zone of inhibition are reported as a mean value, n=3. Error bars represent standard error of the mean (SEM).

3.2.5 Non-β-lactamase hyperproducers, M1 and M52, show reduction in envelope permeability

Hoescht dye (H33342) accumulation was assayed to estimate the effect of the mutations on envelope permeability. Previously, this assay has been tested in other Enterobacteriaceae species such as *E. coli* and *K. pneumoniae* (200, 244). However, non-fermentative bacteria have a peculiarity in terms of permeability when compared to *E. coli*. Solute diffusion in *P. aeruginosa, Burkholderia cepacia, A. baumanii,* and *S. maltophilia* is only 1-11% of that seen for *E. coli* (245). In *S. maltophilia,* this percentage is attributable to a reduced copy number of porins since the porin size is similar to those present in *E. coli* (245, 246). Hence, while the concentration of H33342 used to test permeability in Enterobacteriaceae species was 2.5 µM in other studies (200, 244), here dye concentration was optimised to 25 µM. Cytoplasmic accumulation of the dye in the non-β-lactamase hyperproducers, M1 and M52 when compared to the parental strain K279a, was reduced (**Figure 3.6**). Differences in permeability between M1 and M52 were seen, and this was clearer during growth in a low-osmolarity medium (NB) than in a high-osmolarity medium (MHB). Therefore, downstream experiments were performed using cells grown in NB.

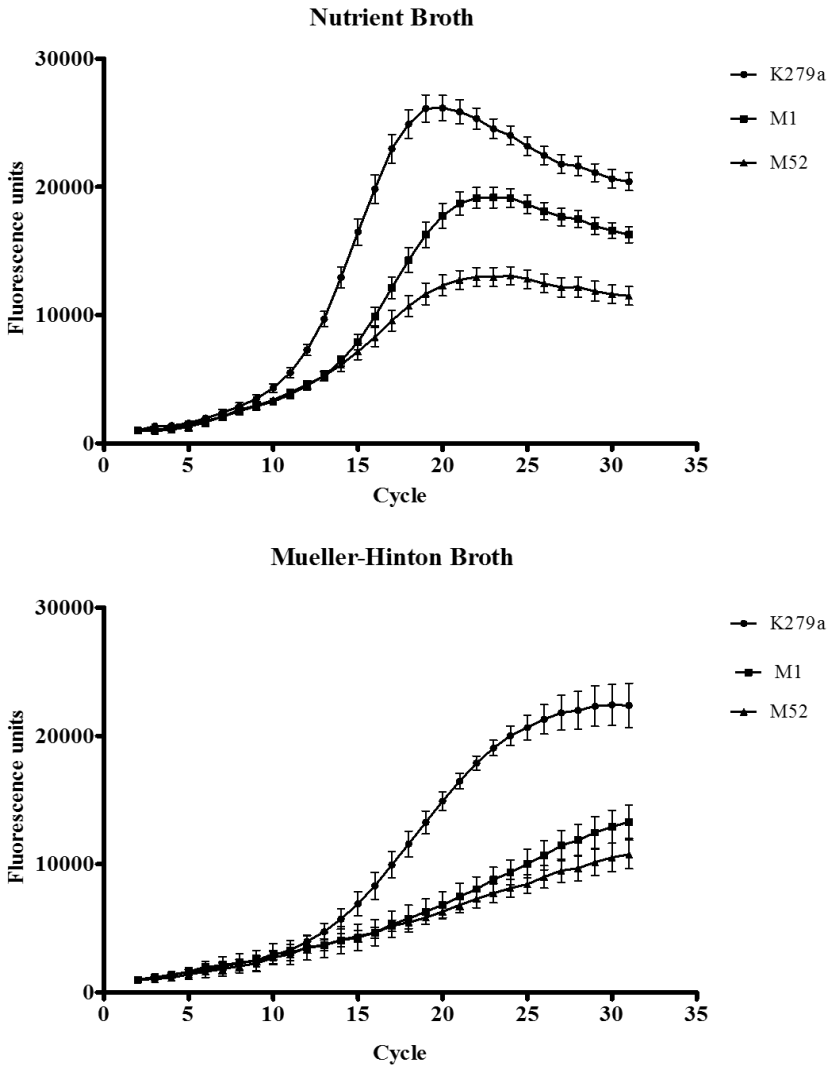


Figure 3.6 Envelope permeability in low-osmolarity (NB) and high-osmolarity (MHB) media, measured using fluorescent dye accumulation

Absolute accumulation of H33342 (25 μM) is expressed as fluorescence units over a 31 cycle incubation period (78 min). Each curve plots mean data for three biological replicates with eight technical replicates. Error bars represent standard error of the mean (SEM)

3.2.6 Disruption of the putative proline-rich TonB energy transducer protein (Smlt0009) causes ceftazidime resistance

Whole genome sequencing was performed to identify the mutations present in the ceftazidime resistant mutants M1 and M52. The only gene found to be mutated in each was *smlt0009* annotated as encoding a 'putative proline-rich TonB energy

transducer protein'. The mutated region is repetitive and short-read technologies such as standard Illumina libraries can be biased to extreme GC-content (247). Therefore, it was decided to re-sequence the gene but this time using a PCR product amplified with Phusion ® High-Fidelity DNA polymerase. Sequences obtained confirmed mutation in *smlt0009* in both mutants M1 and M52 relative to K279a (**Figure 3.7**). To confirm the role of *smlt0009* in ceftazidime resistance, knock-out of the gene was carried out by another member of the group (Punyawee Dulyayangkul) using a suicide gene replacement methodology. K279a Δ*smlt0009* was confirmed to be ceftazidime resistant (disc inhibition zone diameter of 6 mm) when compared to the parent strain, K279a (zone diameter of 27mm).

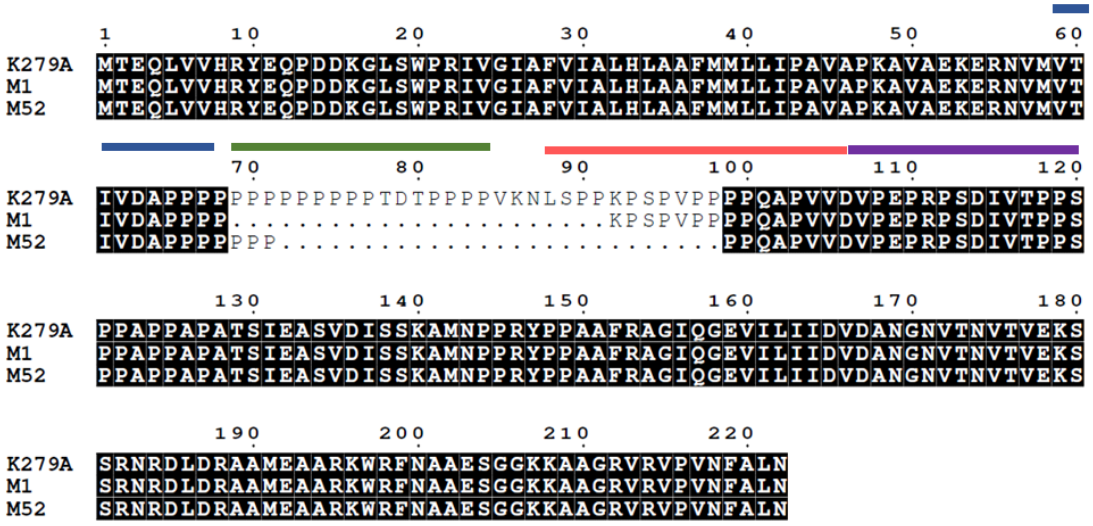


Figure 3.7 Sequence alignment of Smlt0009 putative proline-rich TonB energy transducer of ceftazidime resistant mutants

Alignment of translated PCR sequences that confirmed loss of proline-rich region (non-highlighted region) in M1 and M52 mutants. PCR amplification using Phusion ® High-Fidelity DNA polymerase showing loss of the proline-rich region in the TonB energy transducer. Comparison of proline-rich residues present in TonB *E.coli* with Smlt0009, based on (248), suggests the importance of the proline rich region to achieve the length that allows the energy transducer to spans the periplasm. Blue bar corresponds to 2.9nm, the green bar to 4nm, pink bar 4.6nm and purple bar 3.3nm; the rest of the C-terminus might correspond to 6.5 nm to give a total of ~20nm. Alignment was performed with CLUSTAL Omega and ESPript 3.0.

To understand the mechanism of resistance conferred by this mutation, whole envelope proteomics was performed in M1 and M52 compared with K279a. Proteomics confirmed that the β-lactamases, L1 and L2, are not overporoduced, in M1 and M52 mutants relative to K279a. 162 proteins were identified that are up or down regulated in both M1 and M52 relative to K279a; 83 are downregulated in both and 79 upregulated in both. Within the group of downregulated proteins, the putative proline-rich TonB energy transducer protein Smlt0009 (Uniprot: B2FT87) was 1.8-fold downregulated in M1, and 2.5- fold in M52 when compared to K279a (**Figure 3.9**). Certainly, the mutations seen in Smlt0009 in M1 and M52 are not expected to block production of the protein, but the mutated, presumably inactive, protein may be less stable than wild-type explaining this apparent downregulation of Smlt0009 production in the mutants. Proteomics for K279a Δ*smlt0009* confirmed lack of Smlt0009 (Dulyayangkul, unpublished). Amongst proteins upregulated in M1 and M52 were proteins with the Uniprot accession numbers B2FHQ4, encoded by *entB, smlt2820* (**Figure 3.10**), B2FRE6 (*fepC, smlt2356*) and B2FRE7 (*fepD, smlt2357*). Indeed the entire *smlt2354-7* operon was upregulated (**Annex 1**). These upregulated Fep proteins have been shown to be involved in siderophore production (249). Siderophore production was detected on modified CAS agar, as described in section **2.5**, and found to be increased in M1 and M52, relative to K279a, as predicted from the upregulation of these Fep proteins, as seen in the proteomics (**Figure 3.11**).

Import of siderophores (iron chelating molecules) relies on energy-dependent mechanisms (250). Ferric-siderophore-complex import requires a TonB complex (formed by a proline rich TonB energy transducer protein, ExbB and ExbD) which interacts with one or more of many possible ligand-gated porins (LGPs). The model that describes that interaction is termed ROSET (rotational surveillance and energy transfer). TonB energy transducer proteins have an N-terminus that creates a complex with ExbBD in the inner membrane, a proline-rich region that spans the periplasm and a C-terminus close to the outer membrane. Proton motive force, generated in the inner membrane, is transduced by ExbBD (Smlt0010 and Smlt0011 are the closest homologues in *S. maltophilia*) to cause rotational motion of the N terminus of the TonB energy transducer (Smlt0009). This movement triggers lateral movement on the TonB complex and the TonB C-terminus which surveys the outer membrane to interact with whichever LGP has an exposed TonB box. Ligand binding to an LGP exposes the TonB box. Thus, the TonB energy transducer C-terminus bind to specific LGPs in the presence of their LGP ligands allowing transduction of energy generated in the inner membrane and transformation to kinetic energy to cause a conformational shift in LGP

and ultimately the passage of the ligand into the cell; for example, a siderophore (251) (**Figure 3.8**).

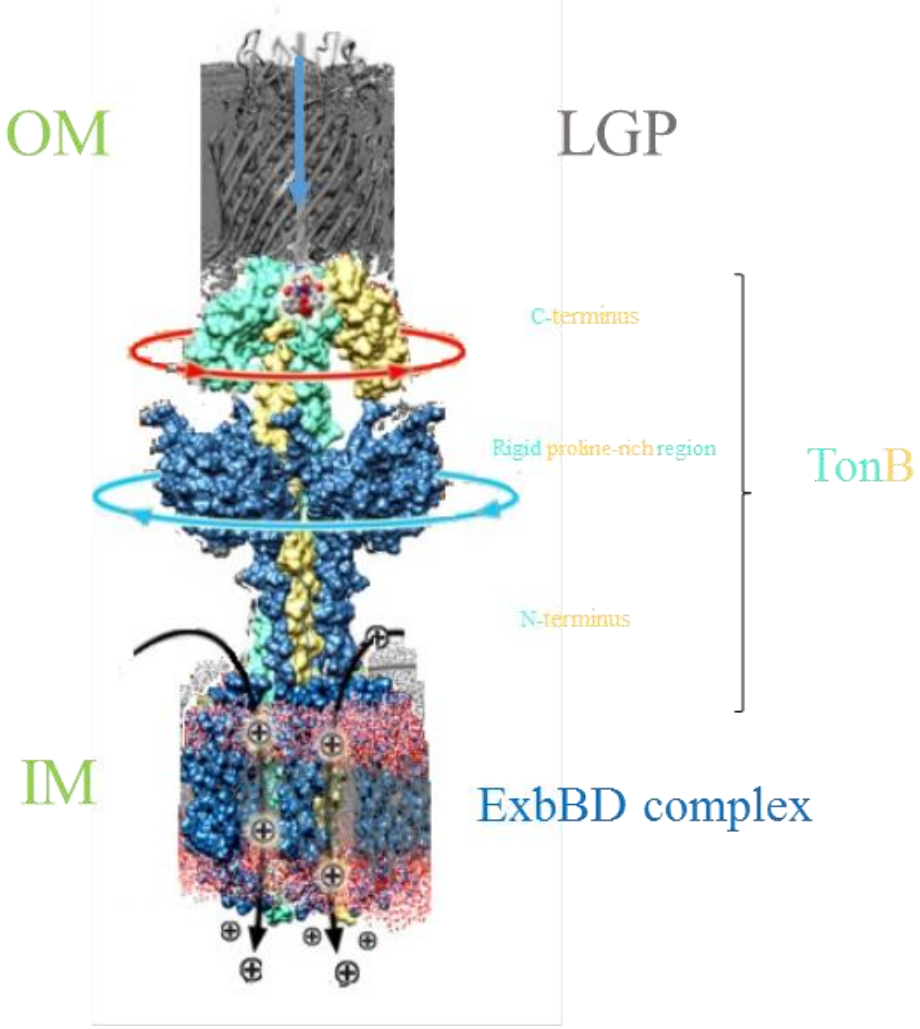


Figure 3.8 Proposed model for TonB dependent uptake

LGPs (ligand-gated porins) embedded in the outer membrane (OM) experiment conformational changes that enable substrate uptake. After substrate binding, the TonB box of the LGP is exposed which allows interaction with the TonB complex (TonB-ExbBD) and consequently transduction of the energy generated in the inner membrane (IM) through proton motive force. Thus ligand binding in the OM and transduction of the energy generated in the IM enable substrate uptake. Modified from (251)

Mutants M1 and M52 have mutations in this proline rich TonB energy transducer protein, Smlt0009, so TonB complex depend import of LGP ligands is likely to be reduced. These mutants also have upregulation of proteins involved in siderophore production, leading to observed enhanced siderophore production. One hypothesis to explain this is that, loss of the TonB energy transducer Smlt0009 impede iron-siderophore uptake, which increases siderophore production due to the resulting iron starvation.

In other bacteria, the proline rich TonB energy transducer has been shown to assume different roles in substrate transport. Due to its ability to interact with different LGPs, TonB complexes participate in import of different substrates ranging from iron/siderophore, vitamin B_{12} to maltodextrines and sucrose. In fact, in the environmental species *Xanthomonas campestris*, only 15% of LGPs are involved in iron/siderophore uptake (252). *S. maltophilia* Smlt0009 TonB energy transducer shares 50% identity with the protein from *X. campestris.* Interestingly, of 162 proteins differently regulated in M1 and M52, 19 are TonB-dependent LGP proteins. Suggesting that mutants are responding to a breakdown in TonB-dependent energy transduction.

In terms of ceftazidime resistance, seen here in M1 and M52, we hypothesise that in *S. maltophilia*, ceftazidime and probably other β-lactams are TonB-dependent substrates. Thus, TonB energy transducer mutations, such as loss of the proline-rich region of Smlt0009 seen in M1 and M52 reduces energy-dependent-ceftazidime uptake due to the inability of the β-lactam specific LGP to open. This is the first time that β-lactam entry via a TonB-dependent mechanism has been proposed in any bacterium. However, it is interesting to note that, unlike all other pathogens studied previously, porin loss has never previously been seen to be involved in β-lactam resistance in *S. maltophilia* which suggests a non-porin mediated import mechanism

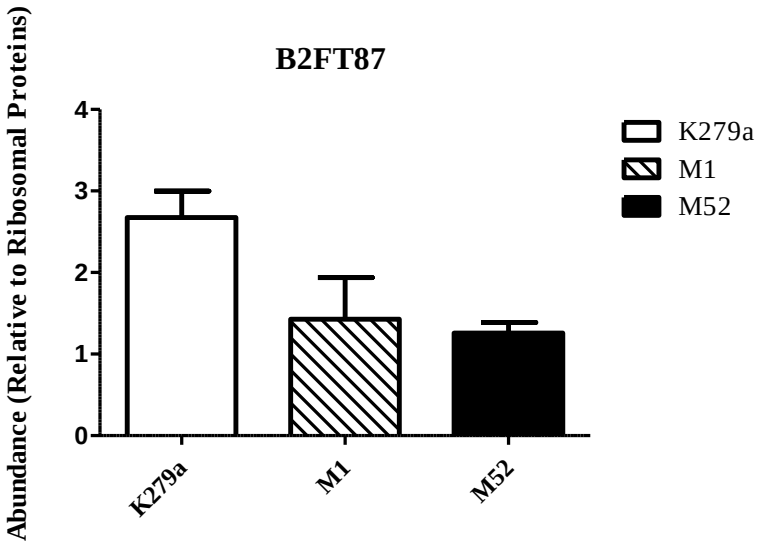


Figure 3.9 Downregulation of the TonB energy transducer protein Smlt0009 in M1 and M52

Protein abundance data of whole envelope proteomics are normalised to the average ribosomal content of each sample (including abundance of 30S and 50S ribosomal proteins). Fold change of Smlt0009 (Uniprot: B2FT87) is calculated after testing statistical significant difference between the parental strain and the mutants (p <0.05). Values are reported as mean +/- Standard Error of the Mean (n=3). Full Proteomics data are shown **Annex 1**

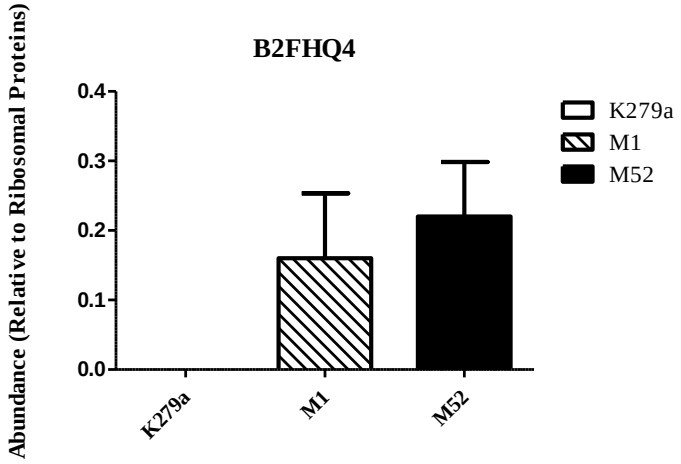


Figure 3.10 Upregulation of EntB siderophore biosynthesis enzyme

Protein abundance data of whole envelope proteomics are normalised to the average ribosomal content of each sample (including abundance of 30S and 50S ribosomal proteins). Fold change of EntB (Uniprot: B2FH84) is calculated after testing statistical significant difference between the parental strain and the mutants (p <0.05). Values are reported as mean +/- Standard Error of the Mean (n=3). Full Proteomics data are shown in **Annex 1.**

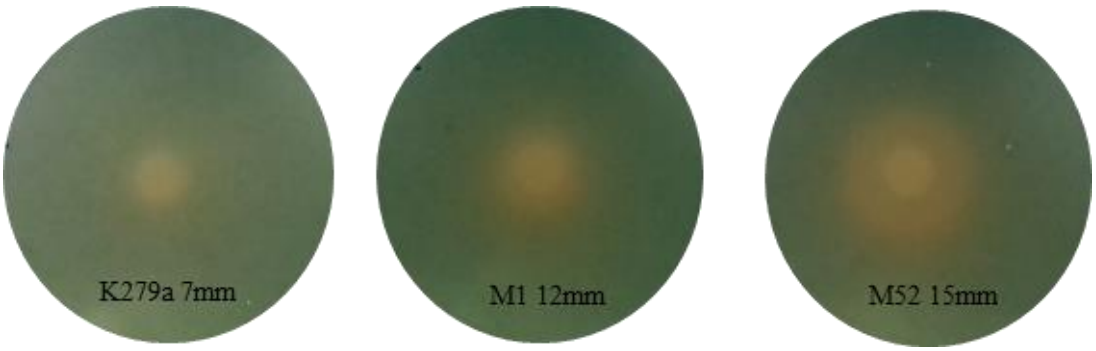


Figure 3.11 Siderophore overproduction of M1 and M52

Diameter values show diffusion of the siderophore production after spotting 10 µL of a PBS washed bacterial suspension (OD_{600} 0.2) onto a modified CAS agar (as described in section **2.4**). Values are reported as mean of three biological repeats; the images are representative

3.2.7 Ceftazidime uptake reduces in non-β-lactamase producers

As stated before, we hypothesised that ceftazidime is a TonB-dependent substrate in *S. maltophilia* and that M1 and M52 do not import ceftazidime at the same rate as K279a, resulting in resistance. To test this, we measured the remaining ceftazidime present in a filtered culture supernatant after exposing K279a, M1 and M52 to ceftazidime (50 µg.mL^{-1} and 25 µg.mL^{-1}). To estimate ceftazidime concentration, we spotted the supernatant onto a lawn of a ceftazidime susceptible *E.coli* laboratory strain (DH5α) spread as if of CLSI disc susceptibility testing. As expected, the residual antimicrobial (ceftazidime) activity in supernatants of M1 and M52 was greater than that in supernatants of K279a cultures. This, demonstrates that K279a takes up more ceftazidime than M1 and M52 (**Figure 3.12**). To add further evidence we tested envelope permeability to the Hoescht dye in the presence of ceftazidime at increasing concentrations. In K279a, permeability reduced in presence of ceftazidime, which means both antibiotic and dye are competing for the same uptake system. This reduction in permeability is not caused by cell death because the concentration of

ceftazidime chosen does not impact on cell growth during mid-exponential phase where cells are harvested to test permeability. OD_{600} reached 0.6 at cycle 49 (**Figure 3.13A**). As previously shown, M1 and M52 are generally less permeable to the dye than K279a, but importantly, in M1 and M52, ceftazidime no longer competes for the dye; i.e. the reduction in permeability seen in the presence of ceftazidime is negligible (**Figure 3.13A**). Therefore, we conclude that the Smlt0009-loss reduces ceftazidime uptake.

	CAZ (4)	K279a (1)	M1 (3)	M52 (2)
50µg.mL^{-1}	28 ±0.33	25 ±0.33	27 ±0.33	27 ±0.33
25µg.mL^{-1}	25±0.33	22 ±0.33	24±0.66	24 ±0.58

Figure 3.12 Inhibition zone(mm) generated by ceftazidime uptake of non-β-lactamase producers against DH5α

K279a, M1 and M52 were incubated in the presence of ceftazidime (50 µg.mL^{-1} and 25 µg.mL^{-1}). Each supernatant was recovered, filtered (0.2 µm) and spotted (10 µL) onto a lawn of *E. coli* DH5α. Zones of inhibition generated, reveal reduced uptake (27 mm) of non-β-lactamase hyperproducers, M1 and M52, when compared to the control, ceftazidime colution, (28 mm). K279a has a superior uptake evidenced by a smaller zone of inhibition (25 mm) meaning a lower ceftazidime concentration in the supernatant. The image shows zone of inhibition generated with ceftazidime (50 µg.mL^{-1}) but the table shows the same tendency for a different concentration of ceftazidime tested (25 µg.mL^{-1}). Values for both concentrations are presented as mean +/- standard error of the mean; the image is representative.

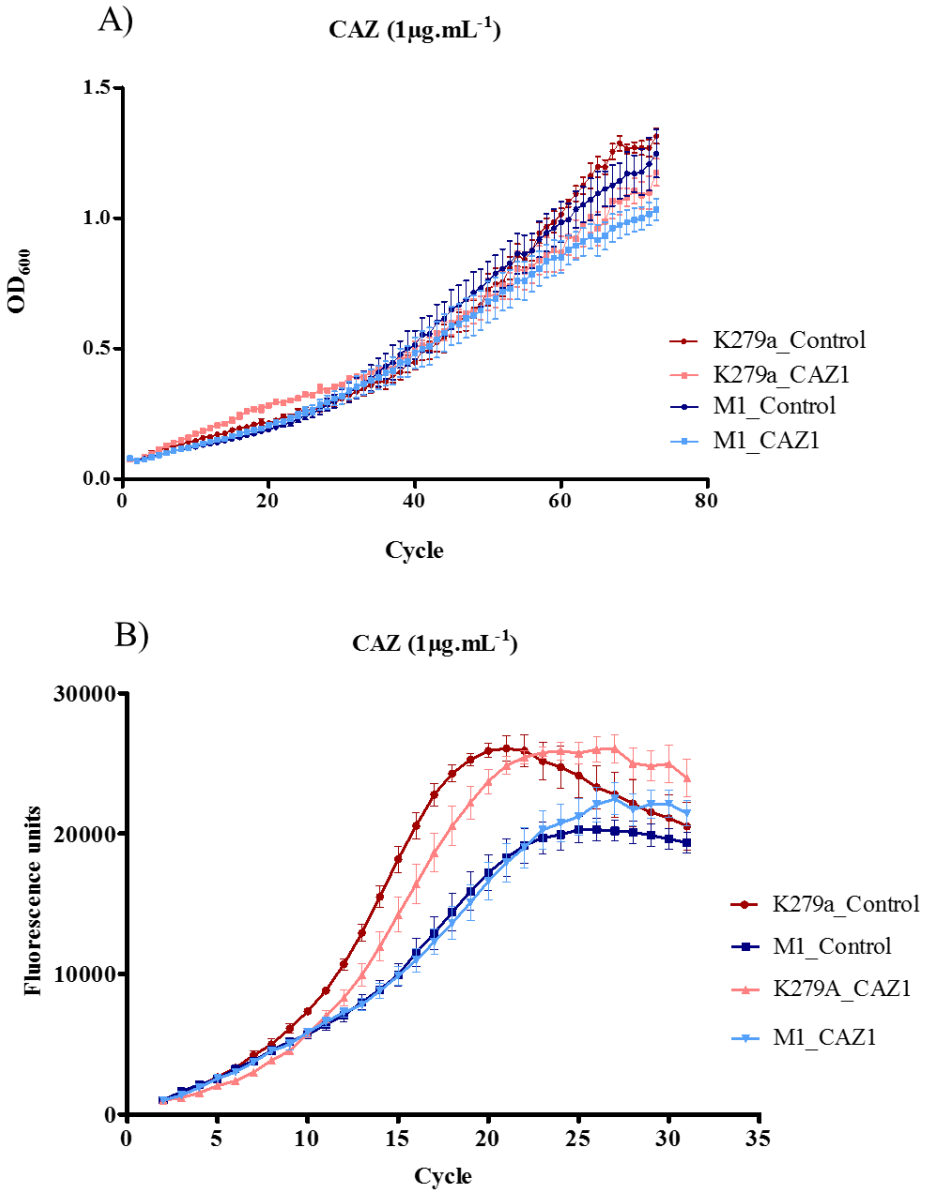


Figure 3.13 Ceftazidime uptake in non-β-lactamase producers responds to changes in permeability

A) Growth curve in NB in absence of ceftazidime (K279a, red and M1, dark blue) and ceftazidime at (1 µg.mL^{-1}) (K279, pink and M1 light blue) over a 73 cycle (12h) incubation period. Growth is conserved during mid-exponential phase **B)** Permeability reduction in presence of CAZ in K279a and maintenance of reduced permeability in M1. Each curve plots mean data for three biological replicates with four technical replicates. Error bars represent standard error of the mean (SEM)

3.2.8 Rate of ceftazidime susceptibility in world-wide collection of *S. maltophilia* clinical isolates is low

In our world-wide collection of 50 clinical isolates, only 10 isolates are susceptible to ceftazidime and within the 40 ceftazidime resistant isolates only four of them are β-lactamase hyperproducers. This would open the possibility to find the mechanism of resistance here proposed where the change of permeability is attributed to the loss of the TonB energy transducer.

3.3 Conclusions

Two novel mutational causes of ceftazidime resistance have been reported. Although the first one described is another method of activating L1 and L2 β-lactamase production, the role of a novel regulatory protein involved in their production has been described. It has been shown that Mpl loss is a clinically important result of β-lactam exposure in *S. maltophilia* and *P. aeruginosa* that emerges as a response to long-term β-lactam exposure, as it happens in cystic fibrosis patients, and ventilator associated pneumonia patients on long-term β-lactam therapy (238, 253).

Characterisation of non-β-lactamase hyperproducers has identified an entirely novel mechanism of ceftazidime resistance that appears to work by reducing the entry of ceftazidime (and other β-lactams but not other classes of antimicrobial) into the cell without affecting the production of porins or efflux pump proteins.

Other work has suggested the possibility of combining ceftazidime and a siderophore group to improve cellular uptake as it happens with cefiderocol (254). However, the improved uptake and hence increase in antimicrobial activity has been mainly attributed to the presence of the siderophore. Here we suggest that the iron uptake systems might not only recognise the siderophore group but instead the structure of ceftazidime. For the first time in bacteria, we have been able to suggest that β-lactam uptake can be TonB-dependent since the phenotype is caused by loss of the TonB energy transducer protein Smlt0009. TonB is frequently involved in iron/siderophore uptake, and indeed there is evidence of this in *S. maltophilia* because Smlt0009 mutants, since siderophore production is enhanced, presumably as an attempt to acquire more iron.

Clinically speaking, this perhaps suggests that Smlt0009-loss mediated ceftazidime resistance is less likely to be a threat, since loss of siderophore uptake

should attenuate virulence. However, in our *S. maltophilia* collection, there are ceftazidime resistant isolates that do not hyper-produce β-lactamase, and it would be interesting to see if Smlt0009 is mutated in any of them.

3.4 Annex 1

Table 3.3 Normalised proteomics data for M1 and M52 relative to K279a

Abundance changes significantly according to a t-Test (P<0.05). Proteins that are >1.5 fold up and down regulated are highlighted green or red, respectively. Proteins are ordered based on Uniprot Accession number. Consecutive numbers are suggestive of operons

Accession	Description	T-Test $_{K279a\ vs\ M1}$	Fold $_{K279a\ vs\ M1}$	T-Test $_{K279a\ vs\ M52}$	Fold $_{K279a\ vs\ M52}$
B2FHA2	Putative ferredoxin oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0136 PE=4 SV=1 - [B2FHA2_STRMK]	0.034	0.42	0.022	0.17
B2FHA8	Putative phospholipase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0142 PE=4 SV=1 - [B2FHA8_STRMK]	0.018	0.53	0.022	0.56
B2FHB5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0149 PE=4 SV=1 - [B2FHB5_STRMK]	0.019	0.53	0.011	0.53
B2FHB7	Glutamine synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=glnA PE=3 SV=1 - [B2FHB7_STRMK]	<0.05	>100	<0.05	>100
B2FHD6	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0170 PE=4 SV=1 - [B2FHD6_STRMK]	0.044	0.62	0.047	0.61
B2FHF0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0184 PE=4 SV=1 - [B2FHF0_STRMK]	0.034	0.58	0.007	0.46
B2FHF9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0193 PE=4 SV=1 - [B2FHF9_STRMK]	<0.05	>100	<0.05	>100
B2FHH2	Putative TonB dependent siderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1426 PE=3 SV=1 - [B2FHH2_STRMK]	0	13.73	0	22.44
B2FHH7	Putative peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1431 PE=4 SV=1 - [B2FHH7_STRMK]	0.004	0.32	0.003	0.3
B2FHJ2	Putative outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1446 PE=3 SV=1 - [B2FHJ2_STRMK]	0.001	0.3	0.001	0.23
B2FHJ5	Putative phosphodiesterase-nucleotide pyrophosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1449 PE=4 SV=1 - [B2FHJ5_STRMK]	0.014	0.47	0.005	0.41
B2FHL6	Putative ABC transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1471 PE=3 SV=1 - [B2FHL6_STRMK]	0.007	0.63	0.05	0.62
B2FHL9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1474 PE=4 SV=1 - [B2FHL9_STRMK]	0.023	0.54	0.014	0.51
B2FHM1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1476 PE=4 SV=1 - [B2FHM1_STRMK]	0.01	0.55	0.018	0.59
B2FHN2	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1491 PE=4 SV=1 - [B2FHN2_STRMK]	0.01	0.37	0.013	0.46

B2FHQ4	Putative hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2820 PE=4 SV=1 - [B2FHQ4_STRMK]	<0.05	>100	<0.05	>100
B2FHT4	Putative TonB dependent extracellular heme-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2850 PE=3 SV=1 - [B2FHT4_STRMK]	<0.05	>100	<0.05	>100
B2FHT9	Putative iron transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2858 PE=3 SV=1 - [B2FHT9_STRMK]	0.003	3.49	0.001	4.77
B2FHX5	Putative CBS domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4098 PE=4 SV=1 - [B2FHX5_STRMK]	<0.05	>100	<0.05	>100
B2FI00	Putative outer membrane Omp family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4123 PE=4 SV=1 - [B2FI00_STRMK]	0.046	0.6	0.012	0.49
B2FI12	Putative colicin I receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=cirA PE=3 SV=1 - [B2FI12_STRMK]	0.001	27.93	0	76.72
B2FI43	Putative peptidyl-dipeptidase Dcp (Dipeptidyl carboxypeptidase) OS=Stenotrophomonas maltophilia (strain K279a) GN=dcp PE=3 SV=1 - [B2FI43_STRMK]	0.032	0.58	0.037	0.58
B2FIA8	Elongation factor Ts OS=Stenotrophomonas maltophilia (strain K279a) GN=tsf PE=3 SV=1 - [EFTS_STRMK]	0.004	1.68	0.033	1.81
B2FIA9	30S ribosomal protein S2 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsB PE=3 SV=1 - [RS2_STRMK]	0.042	0.71	0.015	0.65
B2FIF8	Peptidyl-prolyl cis-trans isomerase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1559 PE=4 SV=1 - [B2FIF8_STRMK]	0.013	1.86	0.001	1.84
B2FII5	Putative CDP-diacylglycerol pyrophosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2903 PE=4 SV=1 - [B2FII5_STRMK]	0.012	0.4	0.006	0.39
B2FIJ2	Putative L-lactate permease OS=Stenotrophomonas maltophilia (strain K279a) GN=lctP PE=4 SV=1 - [B2FIJ2_STRMK]	0.048	1.7	0.013	2
B2FIL8	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2937 PE=3 SV=1 - [B2FIL8_STRMK]	<0.05	>100	<0.05	>100
B2FIN7	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4151 PE=3 SV=1 - [B2FIN7_STRMK]	<0.05	1.22	<0.05	>100
B2FIQ3	Putative lipid A biosynthesis lauroyl acyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=htrB PE=4 SV=1 - [B2FIQ3_STRMK]	0.035	0.62	0.05	0.69
B2FIS6	Protease 4 OS=Stenotrophomonas maltophilia (strain K279a) GN=sppA PE=3 SV=1 - [B2FIS6_STRMK]	0.011	0.54	0.006	0.51
B2FIU9	60 kDa chaperonin OS=Stenotrophomonas maltophilia (strain K279a) GN=groL PE=3 SV=1 - [CH60_STRMK]	0.045	1.7	0.011	1.95
B2FIV0	10 kDa chaperonin OS=Stenotrophomonas maltophilia (strain K279a) GN=groS PE=3 SV=1 - [CH10_STRMK]	0.001	2.49	0.006	2.92
B2FJ60	Putative transmembrane PepSY domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1566 PE=4 SV=1 - [B2FJ60_STRMK]	<0.05	>100	<0.05	>100
B2FJ75	Putrescine-binding periplasmic protein OS=Stenotrophomonas maltophilia (strain K279a) GN=potF PE=3 SV=1 - [B2FJ75_STRMK]	0.018	0.54	0.019	0.58
B2FJ77	Polyamine-transporting ATPase OS=Stenotrophomonas maltophilia (strain K279a) GN=potG PE=3 SV=1 - [B2FJ77_STRMK]	0.021	0.44	0.019	0.46
B2FJB1	Putative oar family adhesion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1619 PE=4 SV=1 - [B2FJB1_STRMK]	0.032	0.6	0.023	0.54
B2FJJ3	Putative hydroxamate-type ferrisiderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3022 PE=3 SV=1 - [B2FJJ3_STRMK]	0.007	12.51	0.002	22.63

B2FJR9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4275 PE=4 SV=1 - [B2FJR9_STRMK]	0.045	1.59	0.027	2.19
B2FJS3	Putative multidrug efflux system HlyD family transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4279 PE=4 SV=1 - [B2FJS3_STRMK]	0.022	0.54	0.013	0.47
B2FJU4	50S ribosomal protein L13 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplM PE=3 SV=1 - [RL13_STRMK]	0.044	1.25	0.004	1.43
B2FJV0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0387 PE=4 SV=1 - [B2FJV0_STRMK]	0.014	0.54	0.002	0.4
B2FK68	Putative glutamate synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1693 PE=3 SV=1 - [B2FK68_STRMK]	0.005	0.38	0.002	0.28
B2FK88	Enolase OS=Stenotrophomonas maltophilia (strain K279a) GN=eno PE=3 SV=1 - [ENO_STRMK]	<0.05	6.86	<0.05	11.58
B2FKE9	Putative P-protein [bifunctional includes: chorismate mutase and prephenate dehydratase OS=Stenotrophomonas maltophilia (strain K279a) GN=pheA PE=4 SV=1 - [B2FKE9_STRMK]	0.022	0.08	0.005	0.07
B2FKJ7	50S ribosomal protein L9 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplI PE=3 SV=1 - [RL9_STRMK]	0.023	1.51	0.015	1.62
B2FKJ9	30S ribosomal protein S6 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsF PE=3 SV=1 - [B2FKJ9_STRMK]	0.003	1.77	0.009	1.67
B2FKL0	Putative haloacid dehalogenase hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4308 PE=4 SV=1 - [B2FKL0_STRMK]	0.05	0.57	0.044	0.58
B2FKM2	Putative K(+)/H(+) antiporter subunit A/B (PH adaptation potassium efflux system protein A/B) OS=Stenotrophomonas maltophilia (strain K279a) GN=phaAB PE=4 SV=1 - [B2FKM2_STRMK]	0.021	1.59	0.028	1.55
B2FKX4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0503 PE=4 SV=1 - [B2FKX4_STRMK]	<0.05	>100	<0.05	>100
B2FL08	Putative transmembrane anchor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0538 PE=4 SV=1 - [B2FL08_STRMK]	0.002	0.25	0.003	0.34
B2FL10	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0540 PE=4 SV=1 - [B2FL10_STRMK]	0.002	0.26	0.002	0.31
B2FL33	Alanine--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=alaS PE=3 SV=1 - [SYA_STRMK]	<0.05	0.9	<0.05	0.68
B2FL51	Putative ferric siderophore receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1762 PE=3 SV=1 - [B2FL51_STRMK]	<0.05	>100	<0.05	>100
B2FL84	Putative succinate dehydrogenase cytochrome b-556 subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=sdhC PE=4 SV=1 - [B2FL84_STRMK]	0.019	0.63	0.005	0.48
B2FL86	Putative succinate dehydrogenase flavoprotein subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=sdhA PE=4 SV=1 - [B2FL86_STRMK]	0.02	0.44	0.006	0.28
B2FL87	Succinate dehydrogenase iron-sulfur subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=sdhB PE=3 SV=1 - [B2FL87_STRMK]	0.021	0.54	0.004	0.36
B2FLB8	Peptidyl-prolyl cis-trans isomerase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3182 PE=4 SV=1 - [B2FLB8_STRMK]	0.038	0.62	0.007	0.44
B2FLD3	Dihydrolipoyl dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=odhL PE=3 SV=1 - [B2FLD3_STRMK]	0.033	1.95	0.017	2.31
B2FLE4	Putative outer membrane antigen protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3210 PE=4 SV=1 - [B2FLE4_STRMK]	0.023	0.59	0.002	0.4
B2FLE9	Putative outer membrane antigen lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3215 PE=4 SV=1 - [B2FLE9_STRMK]	0.034	0.58	0.013	0.51

B2FLG5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3232 PE=4 SV=1 - [B2FLG5_STRMK]	0.025	0.56	0.049	0.64
B2FLQ1	D-amino acid dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=dadA PE=3 SV=1 - [DADA_STRMK]	0.016	0.4	0.016	0.41
B2FLR8	Putative vitamin B12 receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0585 PE=3 SV=1 - [B2FLR8_STRMK]	0.023	0.57	0.018	0.57
B2FLT1	Asparagine synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0598 PE=4 SV=1 - [B2FLT1_STRMK]	<0.05	>100	<0.05	>100
B2FLX9	Putative outer membrane lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1826 PE=3 SV=1 - [B2FLX9_STRMK]	0.03	0.57	0.01	0.49
B2FM92	Ribonuclease E OS=Stenotrophomonas maltophilia (strain K279a) GN=rnE PE=3 SV=1 - [B2FM92_STRMK]	0.003	2.12	0.01	1.92
B2FME8	Putative DNA binding protein/regulator OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3308 PE=3 SV=1 - [B2FME8_STRMK]	<0.05	>100	<0.05	>100
B2FMF2	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3312 PE=4 SV=1 - [B2FMF2_STRMK]	0.04	0.57	0.018	0.51
B2FMI1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4498 PE=4 SV=1 - [B2FMI1_STRMK]	0.025	0.57	0.01	0.55
B2FMN3	Putative transmembrane anchor short-chain dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0632 PE=4 SV=1 - [B2FMN3_STRMK]	0.03	0.62	0.035	0.68
B2FMP6	Putative electron transfer flavoprotein subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=etfS PE=4 SV=1 - [B2FMP6_STRMK]	0.034	2.22	0.001	2.39
B2FMY6	Chaperone protein DnaJ OS=Stenotrophomonas maltophilia (strain K279a) GN=dnaJ PE=3 SV=1 - [DNAJ_STRMK]	0.022	1.66	0.028	3.19
B2FN04	Putative reductase Smlt2015 OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2015 PE=3 SV=1 - [B2FN04_STRMK]	<0.05	2.18	<0.05	2.38
B2FN37	Putative N-acetylmuramoyl-L-alanine amidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3330 PE=4 SV=1 - [B2FN37_STRMK]	0.038	0.56	0.008	0.41
B2FN47	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3340 PE=3 SV=1 - [B2FN47_STRMK]	0.006	0.37	0.003	0.37
B2FNC5	Putative dipeptidyl peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4581 PE=4 SV=1 - [B2FNC5_STRMK]	0.006	0.48	0.014	0.53
B2FND7	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4593 PE=4 SV=1 - [B2FND7_STRMK]	0.003	0.37	0.003	0.45
B2FNF4	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4611 PE=4 SV=1 - [B2FNF4_STRMK]	0.028	0.53	0.009	0.46
B2FNG4	Putative Major Facilitator Superfamily transmembrane transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4621 PE=4 SV=1 - [B2FNG4_STRMK]	<0.05	>100	<0.05	>100
B2FNG5	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4622 PE=4 SV=1 - [B2FNG5_STRMK]	<0.05	5.89	<0.05	5.35
B2FNG6	Acetyl-coenzyme A synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=acsA PE=3 SV=1 - [B2FNG6_STRMK]	<0.05	>100	<0.05	>100
B2FNP5	30S ribosomal protein S1 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsA PE=3 SV=1 - [B2FNP5_STRMK]	0.01	1.72	0.005	1.84
B2FNQ0	Putative epimerase/dehydratase polysaccharide-related biosynthesis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=wbiI PE=4 SV=1 - [B2FNQ0_STRMK]	0.012	0.48	0.027	0.56

B2FNX1	NADH-quinone oxidoreductase subunit I OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoI PE=3 SV=1 - [B2FNX1_STRMK]	0.018	0.48	0.003	0.31
B2FNX6	NADH-quinone oxidoreductase subunit D OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoD PE=3 SV=1 - [NUOD_STRMK]	0.018	0.57	0.004	0.39
B2FNX7	NADH-quinone oxidoreductase subunit C OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoC PE=3 SV=1 - [NUOC_STRMK]	0.011	0.51	0.001	0.33
B2FNX8	NADH-quinone oxidoreductase subunit B OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoB PE=3 SV=1 - [NUOB_STRMK]	0.02	0.51	0.003	0.34
B2FNY0	Putative general secretory pathway protein-export membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=secG PE=4 SV=1 - [B2FNY0_STRMK]	0.001	0.43	0.003	0.52
B2FNY4	Putative lipopolysaccharide core biosynthesis glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3411 PE=4 SV=1 - [B2FNY4_STRMK]	0.04	0.64	0.046	0.67
B2FP19	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3449 PE=4 SV=1 - [B2FP19_STRMK]	0.039	1.64	0.01	2.53
B2FP56	Ferrochelatase OS=Stenotrophomonas maltophilia (strain K279a) GN=hemH PE=3 SV=1 - [B2FP56_STRMK]	0.048	0.5	0.028	0.44
B2FP85	Putative ABC transporter toluene tolerance exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4673 PE=4 SV=1 - [B2FP85_STRMK]	0.005	1.75	0.004	1.76
B2FPA5	Membrane protein insertase YidC OS=Stenotrophomonas maltophilia (strain K279a) GN=yidC PE=3 SV=1 - [YIDC_STRMK]	0.028	0.56	0.014	0.52
B2FPD1	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0784 PE=4 SV=1 - [B2FPD1_STRMK]	0.011	0.45	0.015	0.54
B2FPD9	Probable Na+ dependent nucleoside transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0792 PE=4 SV=1 - [B2FPD9_STRMK]	0.003	0.73	0.032	0.76
B2FPE2	Putative exported heme receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=huvA PE=3 SV=1 - [B2FPE2_STRMK]	0	22.44	0	43.14
B2FPE4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0797 PE=4 SV=1 - [B2FPE4_STRMK]	<0.05	>100	<0.05	>100
B2FPF2	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0805 PE=4 SV=1 - [B2FPF2_STRMK]	0.044	0.6	0.014	0.49
B2FPF9	Prolipoprotein diacylglyceryl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=lgt PE=3 SV=1 - [LGT_STRMK]	0.005	0.44	0.01	0.47
B2FPN1	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2175 PE=3 SV=1 - [B2FPN1_STRMK]	0.009	2.98	0.013	2.65
B2FPQ9	Putative TonB domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3477 PE=4 SV=1 - [B2FPQ9_STRMK]	<0.05	>100	<0.05	>100
B2FPR0	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3478 PE=3 SV=1 - [B2FPR0_STRMK]	0.019	3.83	0.014	4.26
B2FPS0	Putative exported LysM bacterial cell wall related protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3488 PE=4 SV=1 - [B2FPS0_STRMK]	0.02	0.49	0.009	0.43
B2FPS4	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3492 PE=4 SV=1 - [B2FPS4_STRMK]	0.008	0.47	0.012	0.53
B2FPV6	Putative thiolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3525 PE=3 SV=1 - [B2FPV6_STRMK]	<0.05	0.38	<0.05	0.62
B2FPY0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3550 PE=4 SV=1 - [B2FPY0_STRMK]	0.01	0.51	0.04	0.66

B2FPY7	Putative alkyl hydroperoxide reductase subunit c OS=Stenotrophomonas maltophilia (strain K279a) GN=ahpC PE=4 SV=1 - [B2FPY7_STRMK]	0.024	3.37	0.004	4.5
B2FQ28	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0885 PE=3 SV=1 - [B2FQ28_STRMK]	0.016	0.54	0.032	0.59
B2FQ38	DNA-directed RNA polymerase subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoB PE=3 SV=1 - [RPOB_STRMK]	0.037	2.71	0.018	3
B2FQ39	DNA-directed RNA polymerase subunit beta' OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoC PE=3 SV=1 - [RPOC_STRMK]	0.033	2.47	0.022	2.28
B2FQ42	Elongation factor G OS=Stenotrophomonas maltophilia (strain K279a) GN=fusA PE=3 SV=1 - [EFG_STRMK]	0.046	2.16	0.009	1.85
B2FQ45	50S ribosomal protein L3 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplC PE=3 SV=1 - [RL3_STRMK]	0.006	1.37	0.031	1.28
B2FQ48	50S ribosomal protein L2 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplB PE=3 SV=1 - [RL2_STRMK]	0.037	1.57	0.04	1.4
B2FQ50	50S ribosomal protein L22 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplV PE=3 SV=1 - [RL22_STRMK]	0.019	1.77	0.025	2.14
B2FQD3	Putative carbon starvation protein A OS=Stenotrophomonas maltophilia (strain K279a) GN=cstA PE=4 SV=1 - [B2FQD3_STRMK]	0	3.8	0.008	3.55
B2FQJ8	30S ribosomal protein S8 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsH PE=3 SV=1 - [RS8_STRMK]	0.028	0.71	0.031	0.73
B2FQK6	30S ribosomal protein S11 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsK PE=3 SV=1 - [RS11_STRMK]	0.029	1.37	0	1.73
B2FQK8	DNA-directed RNA polymerase subunit alpha OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoA PE=3 SV=1 - [RPOA_STRMK]	0.043	2.11	0.016	1.9
B2FQL8	Malate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=mdh PE=3 SV=1 - [MDH_STRMK]	0.043	2.36	0.034	7.46
B2FQN3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0960 PE=4 SV=1 - [B2FQN3_STRMK]	0.008	0.43	0.007	0.43
B2FQN4	L-threonine 3-dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=tdh PE=3 SV=1 - [TDH_STRMK]	<0.05	>100	<0.05	>100
B2FR08	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3645 PE=3 SV=1 - [B2FR08_STRMK]	0.003	2.73	0.007	3.67
B2FR62	Putative autotransporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1001 PE=4 SV=1 - [B2FR62_STRMK]	0.001	0.1	0	0.09
B2FRC4	Putative TonB-dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1067 PE=3 SV=1 - [B2FRC4_STRMK]	0.003	4.64	0.003	4.28
B2FRE3	Putative esterase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2353 PE=4 SV=1 - [B2FRE3_STRMK]	<0.05	>100	<0.05	>100
B2FRE4	**Putative ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2354 PE=4 SV=1 - [B2FRE4_STRMK]**	<0.05	>100	<0.05	>100
B2FRE5	**Putative binding-protein-dependent transport lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2355 PE=4 SV=1 - [B2FRE5_STRMK]**	0	14.81	0	28.03
B2FRE6	**Putative FecCD-family transmembrane transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2356 PE=4 SV=1 - [B2FRE6_STRMK]**	<0.05	>100	<0.05	>100
B2FRE7	**Hemin import ATP-binding protein HmuV OS=Stenotrophomonas maltophilia (strain K279a) GN=hmuV PE=3 SV=1 - [B2FRE7_STRMK]**	<0.05	>100	<0.05	>100

B2FRZ9	Putative iron transport receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1148 PE=3 SV=1 - [B2FRZ9_STRMK]	0.01	2.17	0.005	2.57
B2FS15	Putative histone-like protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1164 PE=4 SV=1 - [B2FS15_STRMK]	0.02	3.04	0.001	5.72
B2FSE3	Putative HlyD-family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeM PE=4 SV=1 - [B2FSE3_STRMK]	0.027	0.65	0.001	0.39
B2FSE4	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3789 PE=3 SV=1 - [B2FSE4_STRMK]	0.002	5.18	0	4.28
B2FSE9	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3796 PE=4 SV=1 - [B2FSE9_STRMK]	0.002	0.34	0.001	0.34
B2FSF0	Fructose-bisphosphate aldolase OS=Stenotrophomonas maltophilia (strain K279a) GN=alf1 PE=3 SV=1 - [B2FSF0_STRMK]	0.001	2.16	0	2.92
B2FSF6	Glyceraldehyde-3-phosphate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=gap PE=3 SV=1 - [B2FSF6_STRMK]	0.009	2.79	0	2.91
B2FSQ7	Putative TonB-dependent receptor for Fe(III)-coprogen, Fe(III)-ferrioxamine B and Fe(III)-rhodotrulic acid OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1233 PE=3 SV=1 - [B2FSQ7_STRMK]	0	20.43	0	45.94
B2FSS6	Putative PTS system, fructose-specific IIBC component OS=Stenotrophomonas maltophilia (strain K279a) GN=fruA PE=4 SV=1 - [B2FSS6_STRMK]	0.046	1.65	0.034	1.8
B2FST2	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2566 PE=4 SV=1 - [B2FST2_STRMK]	0.019	2.9	0.003	3.07
B2FT59	Putative extracellular heme-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3898 PE=3 SV=1 - [B2FT59_STRMK]	0.006	3.3	0.001	8.38
B2FT68	Putative transcriptional regulator OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3907 PE=4 SV=1 - [B2FT68_STRMK]	0.015	0.5	0.016	0.53
B2FT80	DNA polymerase III subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=dnaN PE=4 SV=1 - [B2FT80_STRMK]	<0.05	>100	<0.05	>100
B2FT86	Conserved hypothetical TPR repeat family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0008 PE=4 SV=1 - [B2FT86_STRMK]	0.001	2.82	0	3.24
B2FT87	**Putative proline-rich TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0009 PE=4 SV=1 - [B2FT87_STRMK]**	0.045	0.53	0.015	0.47
B2FTK5	Putative ferric siderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2650 PE=3 SV=1 - [B2FTK5_STRMK]	<0.05	7.93	<0.05	12.31
B2FTR3	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2712 PE=4 SV=1 - [B2FTR3_STRMK]	<0.05	>100	<0.05	>100
B2FTR4	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2713 PE=4 SV=1 - [B2FTR4_STRMK]	<0.05	>100	<0.05	>100
B2FTR5	Putative TonB dependent protein, possible siderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2714 PE=3 SV=1 - [B2FTR5_STRMK]	<0.05	>100	<0.05	>100
B2FTS4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=pcm PE=4 SV=1 - [B2FTS4_STRMK]	0.025	0.64	0.01	0.49
B2FTS5	Protein CyaE OS=Stenotrophomonas maltophilia (strain K279a) GN=tolC PE=3 SV=1 - [B2FTS5_STRMK]	0.001	0.34	0	0.3
B2FTS8	Putative lipid A biosynthesis lauroyl acyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=htrB PE=4 SV=1 - [B2FTS8_STRMK]	0.045	0.57	0.03	0.55
B2FTY9	Putative endonuclease/exonuclease/phosphatase family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3995 PE=4 SV=1 - [B2FTY9_STRMK]	0.012	0.47	0.006	0.37

B2FU12	Putative iron transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0049 PE=3 SV=1 - [B2FU12_STRMK]	0.015	6.19	0.013	3.74
B2FU42	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0083 PE=3 SV=1 - [B2FU42_STRMK]	0.009	0.45	0.003	0.35
B2FU43	Acid phosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0084 PE=3 SV=1 - [B2FU43_STRMK]	0	0.45	0	0.39
B2FU57	Putative lipoprotein E (Outer membrane protein p4) OS=Stenotrophomonas maltophilia (strain K279a) GN=hel PE=4 SV=1 - [B2FU57_STRMK]	0.029	0.53	0.041	0.61
B2FUD9	Putative HlyD family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1406 PE=4 SV=1 - [B2FUD9_STRMK]	0.033	0.57	0.039	0.63
B2FUL1	Putative respiratory nitrate reductase subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=narH PE=4 SV=1 - [B2FUL1_STRMK]	0.04	0.45	0.018	0.38
B2FUN9	Putative TonB-dependent receptor for Fe(III)-coprogen, Fe(III)-ferrioxamine B and Fe(III)-rhodotrulic acid OS=Stenotrophomonas maltophilia (strain K279a) GN=fhuE PE=3 SV=1 - [B2FUN9_STRMK]	0.001	10.25	0	16.63
B2FUR1	Putative TonB-dependent outer membrane receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4026 PE=3 SV=1 - [B2FUR1_STRMK]	0.05	0.64	0.007	0.37
B2FUV1	Putative multidrug resistance outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeF PE=4 SV=1 - [B2FUV1_STRMK]	0.042	0.65	0.009	0.54

4 Characterisation of new mechanisms of resistance against aminoglycosides, quinolones and tetracyclines in *S. maltophilia*

4.1 Introduction

As stated on the introduction, *S. maltophilia* has been gaining increasing recognition for its intrinsic multidrug resistance which translates into limited therapeutic options. Co-trimoxazole has exhibited the best rate of success against *S. maltophilia* infections (255). However, its administration becomes limited in presence of the sulphonamide-resistant dihydropteroate synthase, in patients who present allergies due to the generation of sulfamethoxazole-specific antibodies caused by the nitroso metabolite of sulfamethoxazole (152) and in situations where intravenous administration is not available. Given the complexity of scenarios that clinicians must face in *S. maltophilia* infections, it becomes imperative to explore therapeutic alternatives beyond co-trimoxazole and with it a deeper understanding of the mechanisms of resistance against the alternatives. Thus, chemotherapy failure can be prevented by increasing the probability of choosing the antibiotic with the greatest potential of success. Apart from the β-lactams, discussed in the previous chapter, aminoglycosides such as amikacin, tobramycin, and gentamicin (152) have been used as part of a combined therapy against *S. maltophilia*. Whilst third and fourth generation quinolones, levofloxacin and moxifloxacin, (256) and the tetracycline derivative minocycline (255) have been used either alone or in a combination with other agents.

Aminoglycosides are normally part of a combined therapy since alone, they show poor activity against *S. maltophilia* infections (257). In fact, mechanisms of resistance against aminoglycoside are wide-ranging. For instance, entry of gentamicin can be temperature-dependent limited due to lipopolysaccharide changes(258). Amikacin efflux can be driven by SmeOP-TolCSm (179) and SmeYZ (259, 260). Alternatively, modifying enzymes such as the AAC (6')-I-type enzyme (116) can acetylate and inactive amikacin. In the case of levofloxacin, monotherapy has shown to be more advantageous than co-trimoxazole concerning side effects for the patient. However, levofloxacin and moxifloxacin have the ability to select resistance at high frequency. Overexpression of SmeJK has been attributed to levofloxacin resistance (260) while upregulation of SmeDEF could explain moxifloxacin-reduced susceptibility(261). Minocycline usage is relatively recent (255) and because of the low number of reported cases of resistance (262, 263), there are few described

mechanisms for minocycline resistance in *S. maltophilia;* on is upregulation of SmeIJK (260).

Previously, two amikacin resistant mutants of *S. maltophilia* K279a have been reported K AMI 8 and K AMI 32. Both have been shown to overexpress the *smeYZ* efflux pump genes, according to RT-PCR and deletion of *smeZ* reverted amikacin resistance (260). Similarly, when trying to unravel the mechanism behind moxifloxacin resistance, two mutants K MOX 2 and K MOX 8 were shown to overexpress the *smeDEF* efflux pump. PCR sequencing revealed a mutation in *smeT,* encoding a transcriptional repressor of *smeDEF* in K MOX 8 but not in K MOX 2, where *smeT* was found to be wild-type (260). Thus, the aims of the work reported in this chapter were to identify the reason for enhanced *smeYZ* expression in K AMI 8 and K AMI 32, and the reason for enhanced *smeDEF* expression in K MOX 2. Furthermore, to select and characterise mutants with reduced susceptibility to levofloxacin and minocycline.

4.2 Results and discussion

4.2.1 Identification of *rplA* mutation in amikacin resistant mutants overexpressing *smeYZ* suggests a novel regulatory mechanism for *smeYZ* expression linking ribosome damage with aminoglycoside resistance

Aminoglycoside resistant mutants K AMI 8 and K AMI 32 were recovered from storage. Antibiotic susceptibility profiling confirmed both mutants retained the previously characteristic phenotype 'resistance profile 3' – particularly reduced zone diameters for aminoglycosides, quinolones and tetracyclines –, as previously reported (260) (**Table 4.1**). Generally, the zone diameters for K AMI 32 were smaller than for K AMI 8. Perhaps explaining this difference, whole envelope proteomics analysis showed a 2-fold upregulation of SmeYZ efflux pump in K AMI 8, and 8-fold upregulation in K AMI 32 relative to the parental strain, K279a (**Figure 4.1**). SmeY is an RND-type efflux protein and SmeZ is a membrane fusion protein. The outer membrane protein that completes the tripartite pump is not known (264). Expression of *smeYZ* is known to be controlled by a two-component system (TCS), SmeSyRy, encoded next to *smeYZ* on the chromosome (180). However, whole genome sequencing (WGS) analysis showed that *smeSyRy* are wild-type in both amikacin mutants, and instead, both have only one mutation that could be identified in the WGS data. In both K AMI 8 and K AMI 32, the

mutation causes a Gly67Asp change in the largest protein from the 50S ribosomal subunit, L1, encoded by the *rplA* gene. A previously described clinical isolate 9189, which also has the aminoglycoside resistance profile 3 and overexpresses *smeYZ (260)* as confirmed in **Table 4.1**, was found by PCR sequencing to carry mutations in *rplA* causing a Phe22Leu change on RplA and a frameshift mutation in codon 212 caused by the insertion of an adenine base in *rplA*. (**Figure 4.2**). In *P. aeruginosa*, MexXY is considered the most important efflux-pump involved in aminoglycoside resistance (265). It has been stated (266) that at least two *P. aeruginosa* clinical isolates that hyperexpress *mexXY* have truncations in *rplA,* though the data were not presented and reported as 'unpublished'. Mutations in other ribosomal subunit genes have more conclusively been shown to cause *mexXY* overexpression, however (265) given the similarities between MexXY and SmeYZ, it seems reasonable to propose the *rplA* disruption is the cause of *smeYZ* overexpression in *S. maltophilia* K279a mutants and the clinical isolate 9189.

Table 4.1 Disc testing profile of parental strain K279a, resistance-profile-3 K279a derivatives and clinical isolate against aminoglycoside, quinolone and tetracycline groups

	Antibiotic (µg)							
	CAZ (30)	**LEV (5)**	**CIP (5)**	**MH (30)**	**CN (10)**	**AK (10)**	**C (30)**	**SXT (25)**
K279a	32	27	23	32	22	24	25	27
K AMI 8	29	20	17	23	11	18	20	23
K AMI 32	29	18	14	25	6	9	23	22
9189	31	24	18	31	6	21	6	6

Shaded values represent reduced zone diameters (≥5mm relative to the parental strain K279a). K AMI 8, K AMI 32, 9189, belong to the resistant profile 3 (reduced susceptibility to aminoglycosides, quinolones and minocycline (189)).

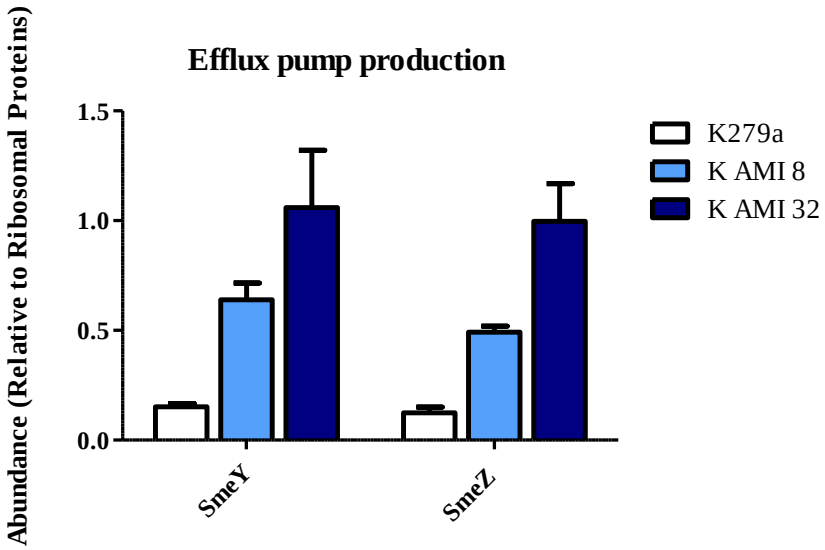


Figure 4.1 Upregulation of efflux-pump SmeYZ proteins in resistance profile 3 mutants derived from K279a

Protein abundance data of whole envelope proteomics are normalised to the average ribosomal content of each sample (including abundance of 30S and 50S ribosomal proteins). Fold change of SmeY and SmeZ (Uniprot: B2FQ54-55) is calculate after testing statistical difference between the parental strain and the mutants (p<0.05). Values are reported as mean +/- Standard Error of the Mean (n=3).

Figure 4.2 RplA amino acid sequence comparison of resistance profile 3 mutants

Alignment of predicted RplA sequences (*smlt0895*) based on PCR sequencing showing the change of well conserved residue Gly67 to Asp in K AMI 8 and K AMI 32 when compared to PDB1U63. A Phe22Leu in the clinical isolate likely due to genetic drift when compared to the sequence of PDB 1U63. Finally a frameshift in the C-terminus of the clinical isolate 9189 (non-highlighted regions). Alignment was performed with CLUSTAL Omega and ESPript 3

4.2.2 Expression of *smeZ* is inducible in presence of sub-lethal concentrations of gentamicin

Again in *P. aeruginosa,* expression of *mexXY* is inducible in response to ribosomal acting agents (267, 268), and since ribosomal protein damage by mutation is associated with *smeYZ* overexpression in *S. maltophilia,* we next tested the inducibility of *smeZ* expression by ribosomal inhibition caused by aminoglycosides. Expression of *smeZ* was assessed by RT-qPCR from a culture of K279a exposed to the aminoglycoside gentamicin (32 µg.mL^{-1}) versus a control culture. Expression of *smeZ* in the control and gentamicin-treated samples were significantly different (p<0.0001) with, as predicted, an increase of *smeZ* expression during gentamicin challenge **Figure 4.3**. In general, induction of the expression of RND efflux pumps is not only

limited to the presence of the efflux-pump substrate but to membrane-damaging agents or ribosomal disruption (269). One of the first pieces of evidence for a nonantibotic substrate for RND efflux-pump was found in *E. coli*. It was proposed that metabolite accumulation due to mutations involved in the disruption of nucleotide or amino acid biosynthetic pathways may lead to active metabolite efflux to avoid a rise in the concentration of toxic metabolic products (270). In *P. aeruginosa,* it has also been proposed that the buil-up of aberrant polypeptides from a damaged ribosome in the presence of ribosome-targeting antibiotics may act as signal for induction of MexXY production (271). This may also be the case in *S. maltophilia* for SmeYZ production. Here, we have confirmed that ribosomal damaging aminoglycosides activate production of an aminoglycoside efflux pump in *S. maltophilia* and suggest the signal is direct ribosomal damage, as for MexXY induction in *P. aeruginosa* (271). Loss of SmeYZ negatively impacts virulence. Whils flagella and biofilm formation decrease, susceptibility to human neutrophils increases (259).Thus, as well as affecting aminoglycoside susceptibility, increases in *smeYZ* expression might increase virulence, in fact when we checked swimming motility in K AMI 8 and K AMI 32 (data not shown), both strains presented more motility when compared to the parental strain K279a.

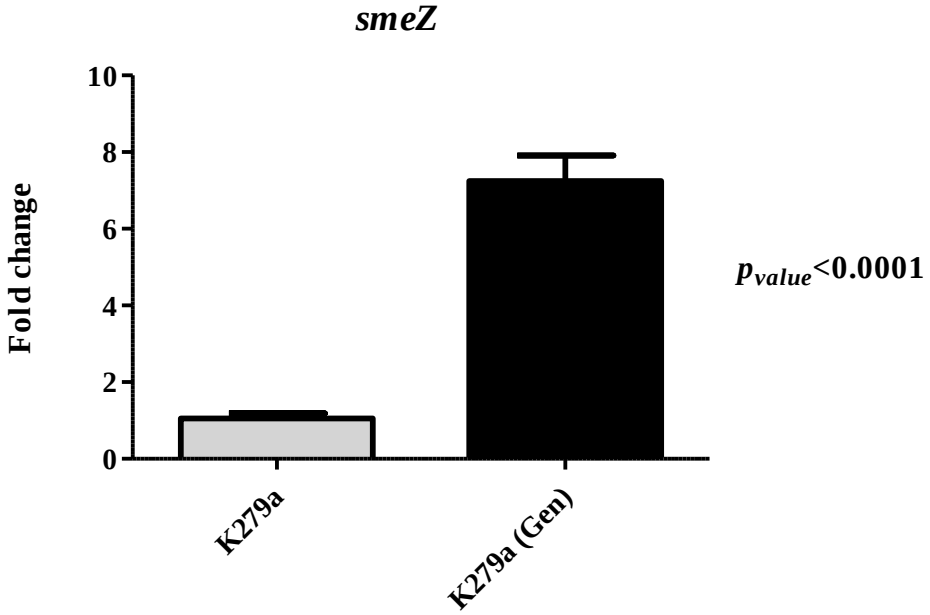


Figure 4.3 RT-qPCR analysis of effect of gentamicin on *smeZ* expression

A control and an experimental K279a cultures were grown in NB for 3 h.

Experimental group was grown in presence of gentamicin (32 µg.mL^{-1}). Fold change of *smeZ* expression between control and experimental group was calculated using the 2^ΔΔCt method and statistical difference between groups was calculated using a two-tailed, unpaired T-Test in GraphPrism (p<0.0001)

4.2.3 Reciprocal relationship between *smeDEF* and *smeYZ* in resistance profile 1 mutants is confirmed by whole envelope proteomics

The 'resistance profile 1' mutants K MOX 2 and K MOX 8 were also recovered from storage and confirmed to have the expected profile of resistance – decreased ciprofloxacin and chloramphenicol susceptibility- (189) (**Table 4.2**). Whole envelope proteomics analysis confirmed the previously reported overexpression of *smeDEF* in these two mutants (189). There was a 1.5-fold upregulation of SmeDEF in K MOX 2, and 3-fold upregulation of SmeDEF in K MOX 8 relative to the parental strain, K279a (**Figure 4.4**). Again, the increased amount of SmeDEF in K MOX 8 versus K MOX 2 may explain the fact that susceptibility is reduced more in K MOX 8 than K MOX 2 relative to K279a (**Table 4.2**). K MOX 2 and K MOX 8 were previously shown to have increased susceptibility to amikacin when compared to resistance profile 3 mutants (189) (**Table 4.1**) (**Table 4.2**) . Comparative proteomics confirmed that in K MOX 2 and K MOX 8, SmeYZ was downregulated as SmeDEF was hyperproduced, which would explain why aminoglycoside susceptibility increases in K MOX 2 and K MOX 8 as part of resistance profile 1. Interestingly, it has recently been reported that overexpression of *smeYZ* leads to reduced *smeDEF* expression (259) suggesting a reciprocal regulation of these pumps in both directions. However, our proteomics showed that SmeDEF production was not reduced in the SmeYZ over-producing mutants K AMI 32 and K AMI 8, so this regulation was only in one direction in our hands. (**Figure 4.4**).

Table 4.2 Disc testing profile of parental strain K279a and resistance-profile-1 K279a derivatives against aminoglycoside, quinolone and tetracycline groups

	Antibiotic (µg)							
	CAZ (30)	**LEV (5)**	**CIP (5)**	**MH (30)**	**CN (10)**	**AK (10)**	**C (30)**	**SXT (25)**
K279a	32	27	23	32	22	24	25	27
K MOX 2	30	19	13	27	22	25	23	22
K MOX 8	31	18	14	27	21	23	22	22

Shaded values represent reduced zone diameters (≥5mm relative to the parental strain K279a). K MOX 2, K MOX 8, belong to the resistant profile 1 (susceptible to aminoglycosides, but resistant to quinolones and minocycline (189)).

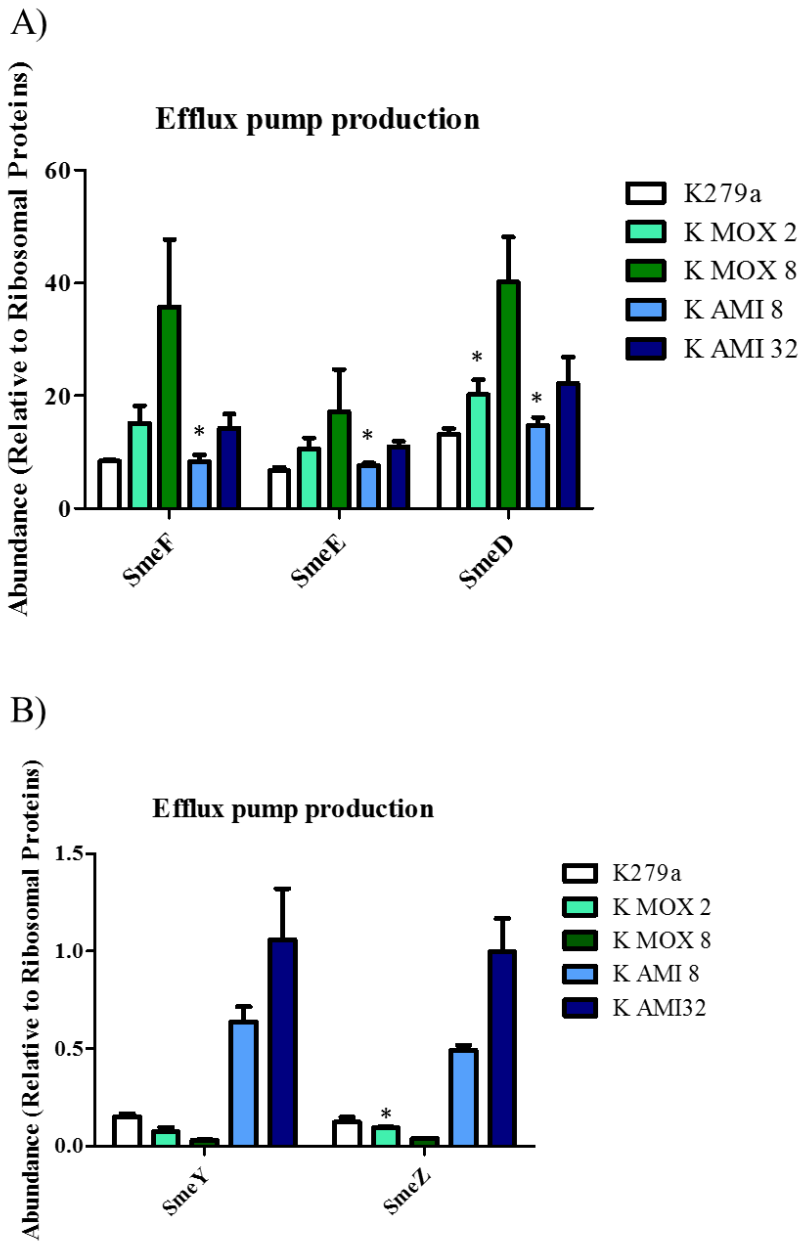


Figure 4.4 Reciprocal relationship of *smeDEF* and *smeYZ* expresssion

Protein abundance data of whole envelope proteomics are normalised to the ribosomal content of each samples (including abundance of 30S and 50S proteins). Fold change was calculated after testing statistical difference between the parental strain and the mutants (p <0.05). p values >0.05 are marked with (*). **A)** Upregulation of efflux-pump SmeDEF in resistance profile 1 (KMOX2 and KMOX8) and profile 3 (KAMI8 and KAMI32) mutants. **B)** Upregulation of efflux-pump SmeYZ in K MOX 8 but not in the opposite way in K AMI 32. Values are reported as mean +/- Standard Error of the Mean (n=3). Full Proteomics data for K MOX 8 and K AMI 32 are shown in (272).

The reason behind overexpression of *smeDEF* in K MOX 8 is a point mutation that disrupts the local repressor of *smeDEF*, SmeT (189). However, K MOX 2 conserved a wild-type *smeT* which suggested the involvement of a different regulatory mechanism for *smeDEF* expression and the authors proposed that 'It may be that the mutated regulatory locus in K-M6 (also known as K MOX 2) encodes a protein whose function is to regulate the concentration of a ligand that signals for SmeT to derepress *smeDEF* expression'. WGS was performed on K MOX 2 and revealed a single mutation relative to K279a predicted to cause a Gly368Ala change in a putative glycosyltransferase encoded by the *wxocA* gene (**Figure 4.5**). Gly368 is a particularly well-conserved residue when searching in the *S. maltophilia* genomes in the Genbank database since no alternative residues were found at that position. Glycosyltransferases are responsible of the addition of saccharides onto other biomolecules. Therefore they can utilize various substrates and participate in myriad cellular functions. For example, synthesis of the core oligosaccharide region of the lipopolysaccharide (273) cellular detoxification, solubilisation of substrates (274). Currently, there is no information about the specific role of the glycosyltransferase encoded by *smlt0622*. However, the *wxocA* of a *Xanthomonas oryzae* strain has been reported to have 62% identity and 74% similarity to a homologous lipopolysaccharide biosynthesis protein-like protein [Uniprot A5KT55] (275). When we compared the WxocA sequence of K MOX 2 to A5KT55, we found 52% identity and 94% similarity. But again, without knowing its exact cellular role, it only seems possible to suggest why the alteration found in this particular glycosyltransferase is a reason for constitutive activation of SmeDEF. It may be that the earlier publication is correct and that the glycosyltransferase is involved in cellular detoxification, and that loss of this detoxification leads to the build-up of a toxic chemical that activates SmeDEF production, and is a substrate for this pump. However, this remains to be seen.

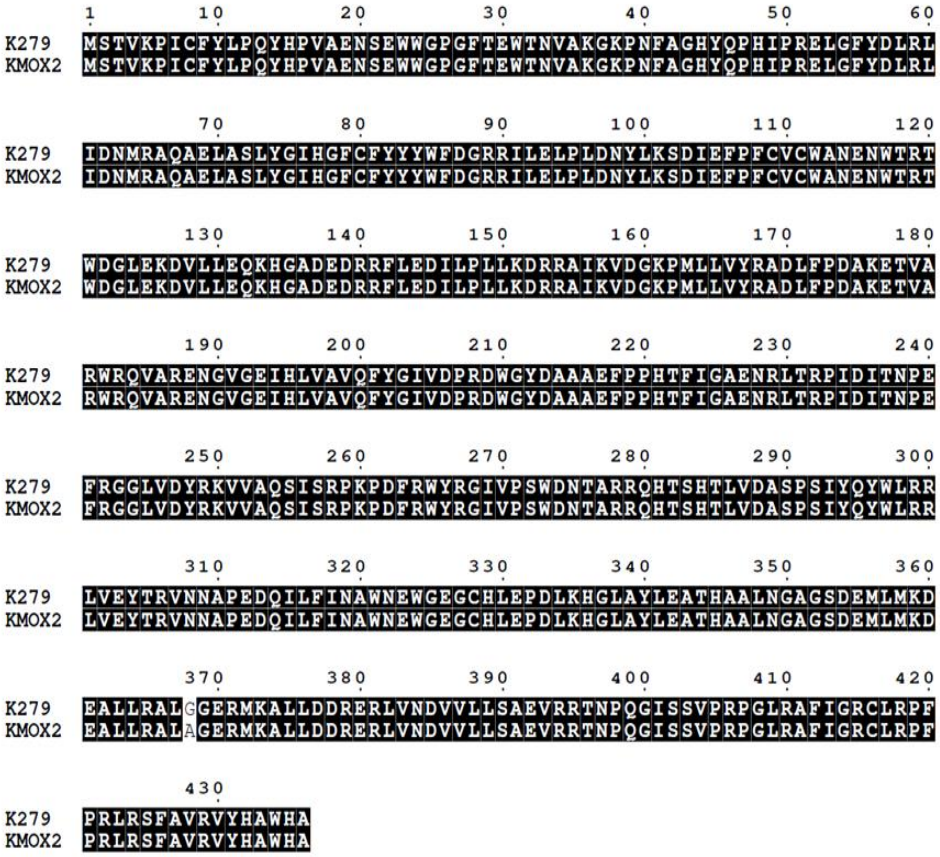


Figure 4.5 Glycosyltransferase amino acid sequence comparison of type 1 resistant mutants

Alignment of glycosyltransferase (*wxocA*) sequences showing Gly368Ala change in K MOX 2 which is a well conserved residue (non-highlighted regions) which is a well conserved residue. Alignment was performed with CLUSTAL Omega and ESPript 3.0

4.2.4 Characterisation of a novel mechanism of enhanced fluoroquinolone resistance caused by a bipartite ABC transporter, regulated by a divergent two-component system

Next, we attempted to characterise mechanisms that cause resistance to the fluoroquinolone levofloxacin, which is showing promise for treatment of *S. maltophilia* infections (256, 276). When trying to select a single-step-levofloxacin resistant mutant from K279a, no mutant was recovered. Thus, K LEV 5 was obtained as a second-step-levofloxacin resistant mutant derived from K MOX 2, which whilst being moxifloxacin

resistant, retains levofloxacin susceptibility. K LEV 5 presented a similar resistance profile to K MOX 2, but with further reduced inhibition zones for ciprofloxacin and levofloxacin according to disc testing (**Table 4.3**). Whole envelope proteomics analysis revealed upregulation of a bipartite ABC transporter (annotated as MacAB based on its similarity to this well characterised pump from *E. coli* (173, 277) in K LEV 5 compared with K MOX 2. However, since there is no evidence for this being a macrolide efflux pump in *S. maltophilia* we will refer to this putative efflux pump by its Smlt number (Smlt2642-3). We also noticed in the proteomics data that a two-component system (Smlt2645-6), encoded immediately adjacent Smlt2642-3 is also overproduced in K LEV 5 relative to K MOX 2 (**Annex 2**). According to WGS K LEV 5 has only one mutation, predicted to cause an Ala198Thr change in the sensor histidine kinase protein (Smlt2646) (**Figure 4.6**). This histidine kinase is therefore a good candidate for local activation of this ABC transporter given its location in the chromosome. Since the output of a two-component system is dominant due to amount of the phosphorylated regulator, we aimed to confirm the effect of the mutated version of *smlt2646* in a wild-type background, *in trans*. The operon, including the response regulator and the histidine kinase (*smlt2644-smlt2646*), was cohesive-end ligated into pBBRMCS-4 (Amp^R) (235, 236) and the recombinant plasmid was used to transform. K279aAmp^{FS}, which is an ampicillin susceptible derivative of K279a (160) to ampicillin resistance (100 µg.mL^{-1}) via electroporation. To confirm the insertion of a unique insert we digested the plasmid pBBRMCS-4:: *smlt2644-6* with EcoRI and observed the presence the ~2709bp-expect insert (**Figure 4.7**). Disc testing showed a reduction in levofloxacin susceptibility in K279aAmp^{FS} bearing pBBR1MCS-4::*smlt2644-6* relative to the plasmid only control transformant. An original zone of inhibition around a levofloxacin disc of 37 mm decreased to 20 mm in the recombinant (**Figure 4.8**). Accordingly, this is an entirely novel quinolone resistance mechanism in *S. maltophilia*.

Table 4.3 Disc testing profile of initial parental strain K279a and resistance-profile-1 K MOX 2 and its derivative K LEV 5against aminoglycoside, quinolone and tetracycline groups

	Antibiotic (µg)							
	CAZ (30)	**LEV (5)**	**CIP (5)**	**MH (30)**	**CN (10)**	**AK (10)**	**C (30)**	**SXT (25)**
K279a	32	27	23	32	22	24	25	27
K MOX 2	30	19	13	27	22	25	23	22
K LEV 5	30	14	11	27	20	22	22	22

Shaded values represent reduced zone diameters (≥5mm relative to the parental strain K279a). K MOX 2 and K LEV 5, belong to the resistant profile 1 (susceptible to aminoglycosides, but resistant to quinolones and minocycline (189)).

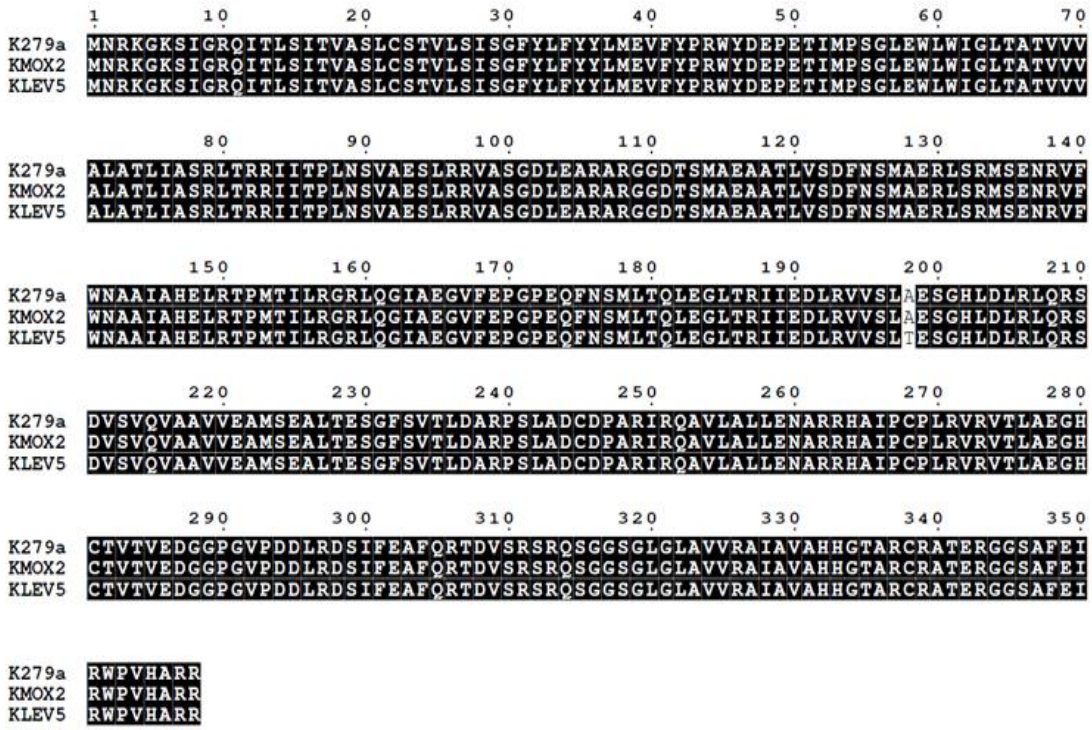


Figure 4.6 Histidine kinase amino acid sequence comparison of type 1 resistant mutants

Alignment of histidine kinase (*smlt2646*) sequences showing Ala198Thr change in K LEV 5 (non-highlighted regions) which is a well conserved residue and its mutation might interrupt signal transduction since this region is likely to correspond to a cytoplasmic linker . Alignment was performed with CLUSTAL Omega and ESPript 3.0

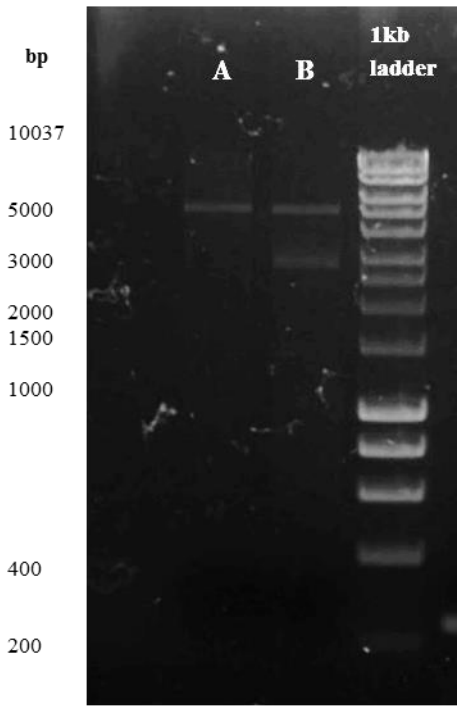


Figure 4.7 Enzymatic digestion of the vector containing the operon containing the two-component system (Smlt2644-6) during complementation *in trans*

A) Linearized vector with EcoRI from K279a *ampR*^{FS} carrying pBBR1MCS-4 (4950bp) **B)** Digested vector where backbone corresponds to 4950bp and the insert (*smlt2644-46)* to 2709bp from K279a *ampR*^{FS} carrying pBBR1MCS-4::*smlt2644-46.* To improve visualisation, brightness has been modified.

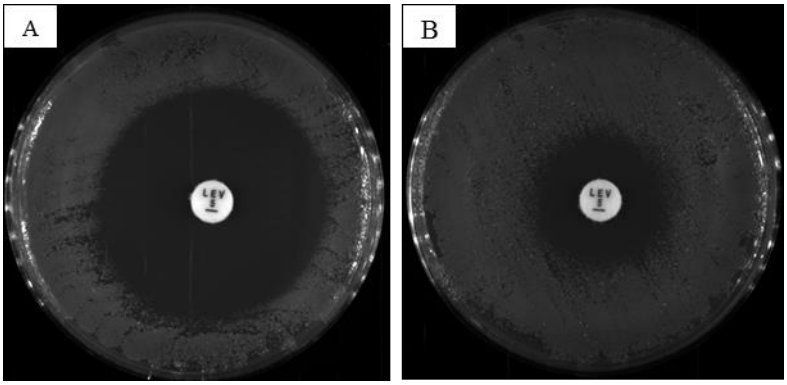


Figure 4.8 Expression in *trans* of two-component system (Smlt2644-6) in K279a AmpR^{FS} reduces susceptibility to levofloxacin

A) K279a *ampR*^{FS} carrying pBBR1MCS-4: zone of inhibition 37 mm **B)** K279a *ampR*^{FS} carrying pBBR1MCS-4::two-component system 20 mm

4.2.5 Rate of levofloxacin susceptibility in world-wide collection of *S. maltophilia* clinical isolates

Levofloxacin resistance in the collection of *S. maltophilia* clinical isolates only corresponds to 32% according to the CLSI guidelines (197). Hence, it would be interesting to test how common are our findings of ABC transporter upregulation are in clinical isolates.

4.2.6 Mutants with reduce susceptibility to minocycline overproduce an ABC transporter, Smlt1640, regulated by a divergent two-component system

Minocycline is another antimicrobial with potential against *S. maltophilia* infections and rates of resistance are very low. In fact, in our world-wide collection all clinical isolates were susceptible to minocycline, according to the CLSI breakpoints (197) (**Table 4.4**) .

Table 4.4 Zone diameters (mm) around minocycline discs for worldwide collection of *S. maltophilia* clinical isolates (C.A.) All isolates are susceptible to minocycline (MH). Susceptibility breakpoint ≥ 19 mm.

	MH (30 µg)
K279a	32
C.A.1	33
C.A. 2	33
C.A. 3	20
C.A. 4	28
C.A. 7	33
C.A. 8	35
C.A. 9	31
C.A. 10	34
C.A. 11	31
C.A. 12	28
C.A. 13	32
C.A. 14	29
C.A. 15	27
C.A. 16	29
C.A. 17	29
C.A. 18	33
C.A. 19	30
C.A. 20	33
C.A. 21	31
C.A. 22	31
C.A. 23	35
C.A. 24	30
C.A. 26	32
C.A. 27	27

	MH (30 µg)
C.A. 28	27
C.A. 29	31
C.A. 30	26
C.A. 31	28
C.A. 32	30
C.A. 33	31
C.A. 34	30
C.A. 35	30
C.A. 36	32
C.A. 37	32
C.A. 38	30
C.A. 39	31
C.A. 40	30
C.A. 41	30
C.A. 42	31
C.A. 43	30
C.A. 44	26
C.A. 45	31
C.A. 46	31
C.A. 47	34
C.A. 49	29
C.A. 50	29
C.A. 51	32
C.A. 52	27
ULA511	30

In order to understand how minocycline resistance might arise we attempted to select minocycline resistant mutants. We failed to find colonies that grew above the reported breakpoint for minocycline resistance, and the highest MIC seen was 2mg.L^{-1} against a mutant selected via two steps from K279a, named K MIN 2. According to disc testing, K MIN 2 expresses a resistance phenotype similar to the fluoroquinolone resistant mutants (resistance profile 1) but with a slightly smaller zone of clearing for minocycline (**Table 4.5**). Whole envelope proteomics analysis results showed a 4-fold upregulation of SmeDEF, relative to K279a, which explains the general resistance profile of the isolate. However, in addition, we identified a 1.8-fold upregulation of a putative ABC efflux protein annotated as MsbA (Smlt1640) (**Figure 4.9**) (**Annex 3**), which, in other Gram-negative bacteria, contains two different sites for substrate binding. One binding site is involved in transport of lipid A, essential for outer membrane integrity, and the other can interact with drugs which suggests a physiological role for MsbA as well as multidrug transport (278). When compared to K279a genome, WGS showed a Gly115Asp change in a lauroyl acyltransferase, encoded by *smlt4167* (data not shown), and an Arg15Cys change in a histidine kinase part of a two-component system immediately next to a 10-component operon where *msbA* (*smlt1640*) is present (**Figure 4.10**). Many of the other members of this operon are also upregulated in K MIN 2. In order to test involvement of this mutation in the two-component regulator in decreased minocycline susceptibility, we expressed the mutated version of the operon containing the two component system (*smlt1635-smlt1636*) in K279a *ampR*^{FS} as before. Disc testing confirmed reduction in minocycline susceptibility in K279a *ampR*^{FS} carrying pBBR1MCS-4 with the two-component system genes (35mm) relative to K279a *ampR*^{FS} carrying vector only (39mm) (**Figure 4.12**). This suggest an entirely novel, albeit weak, participant in minocycline reduced susceptibility in *S. maltophilia*. However, it is not the first report of an ABC transporter being involved in lipid trafficking to be linked to susceptibility to tetracyclines. In *E. coli,* loss of the *mlaFEDCB* operon, an ABC- dependent participant in the preservation of outer membrane lipid asymmetry (279), leads to accumulation of phospholipids in the outer leaflet of the outer membrane. When Mla is upregulated by mutation, this reduces outer membrane permeability to hydrophobic substances such as minocycline (280). It may be that the *S. maltophilia* ABC transport system reported here in *S. maltophilia* does not export minocycline, but reduces its entry by altering lipid balance.

Table 4.5 Disc testing profile (inhibition zone diameters in mm) of initial parental strain K279a and resistance-profile-1 derivative with reduced susceptibility to minocycline against aminoglycoside, quinolone and tetracycline groups

	Antibiotic (µg)							
	CAZ (30)	**LEV (5)**	**CIP (5)**	**MH (30)**	**CN (10)**	**AK (10)**	**C (30)**	**SXT (25)**
K279a	32	27	23	32	22	24	25	27
K MIN 2	32	18	15	25	25	26	23	20

Shaded values represent reduced zone diameters (≥5mm relative to the parental strain K279a). K MIN 2 belongs to the resistant profile 1 (susceptible to aminoglycosides, but resistant to quinolones and minocycline).

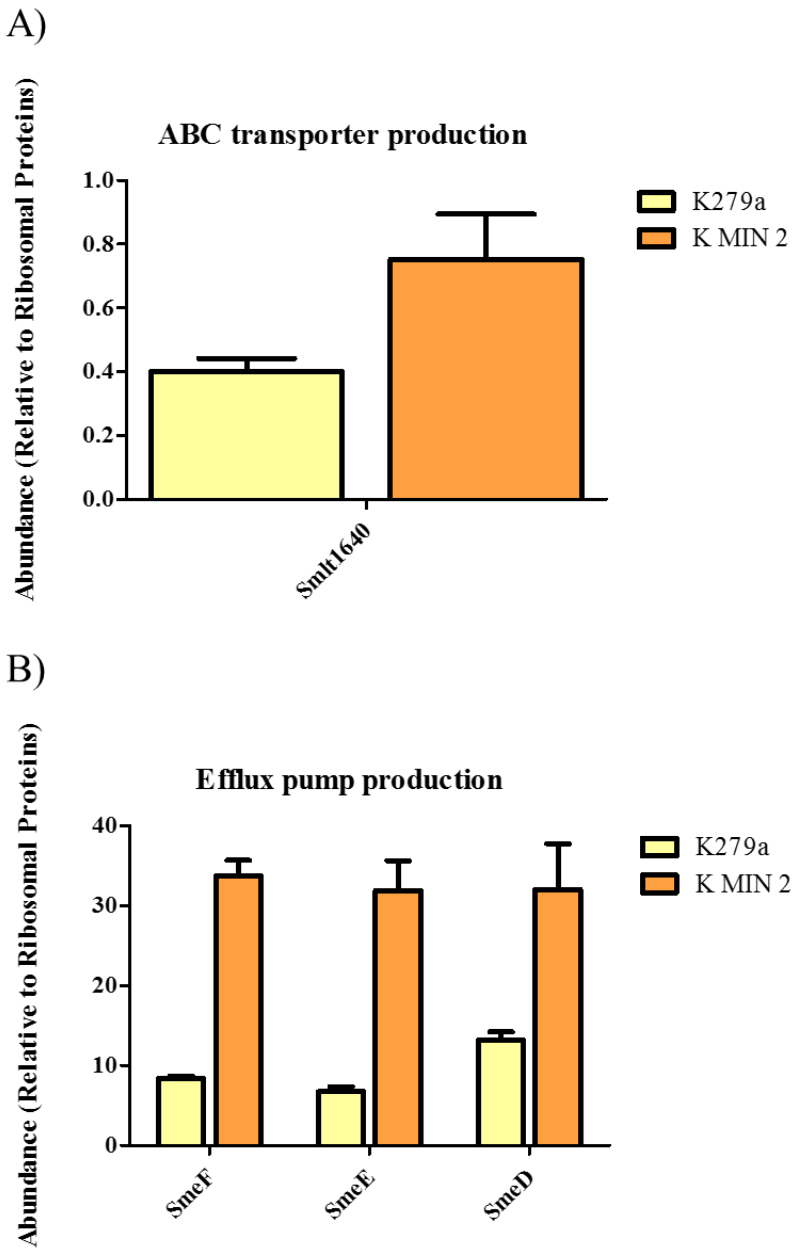


Figure 4.9 Upregulation of A) Smlt1640 B) SmeDEF in minocycline resistant mutant

Protein abundance data of whole envelope proteomics are normalised to the average ribosomal content of each sample (including abundance of 30S and 50S ribosomal proteins). Fold change of **A)** Smlt1640 (Uniprot: B2FK21) and **B)** SmeDEF is calculated after testing statistical significant difference between the parental strain and the mutants (p <0.05). Values are reported as mean +/- Standard Error of the Mean (n=3). Full Proteomics data are shown in **Annex 3**.

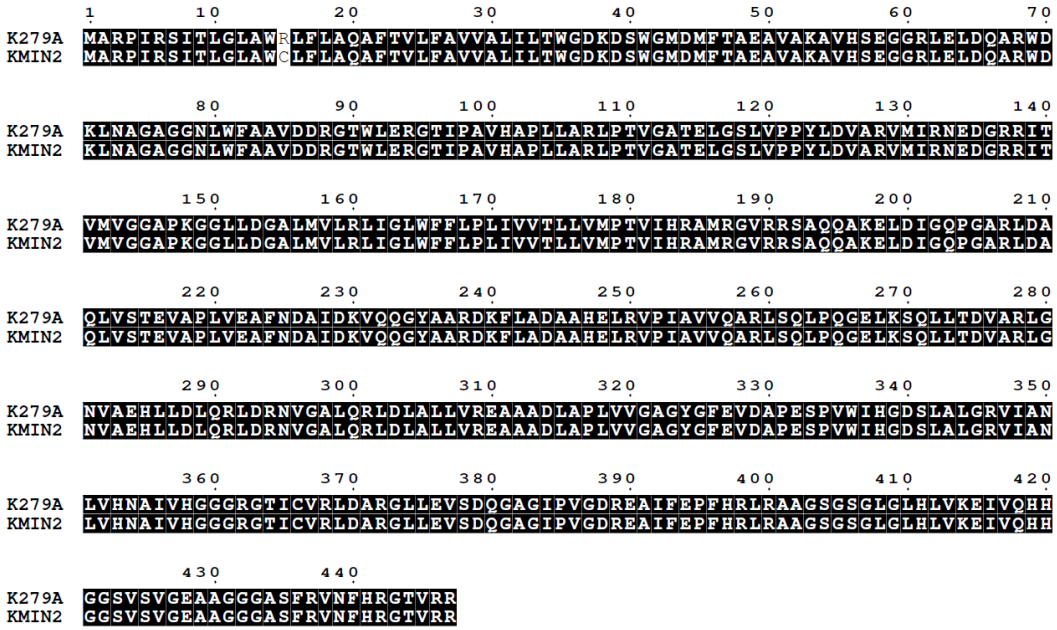


Figure 4.10 Histidine kinase amino acid sequence comparison of minocycline resistant mutants

Alignment of histidine kinase (*smlt1636*) sequences showing Arg15Cys change in K MIN 2 (non-highlighted regions). Alignment was performed with CLUSTAL Omega and ESPript 3.0

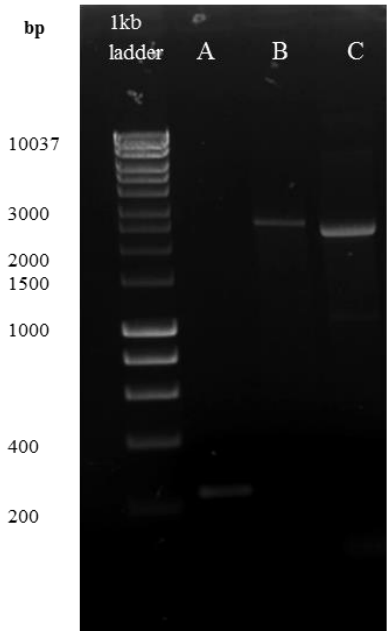


Figure 4.11 *smlt1635-6* (two-component system)products obtained during complementation *in trans*

A) Amplification of the flanking region using M13F and M13R primers corresponding to ~250bp from K279a (*ampR*^{FS}) carrying pBBR1MCS-4 **B)** Amplification of the flanking region using M13F and M13R primers corresponding to ~2700bp from K279a (*ampR*^{FS}) carrying pBBR1MCS-4::*smlt1635-6* **C)** Amplification of *smlt1635-6* using primer Smlt1636_F and Smlt1636_R corresponding to 2305 bp from K279a (positive control)

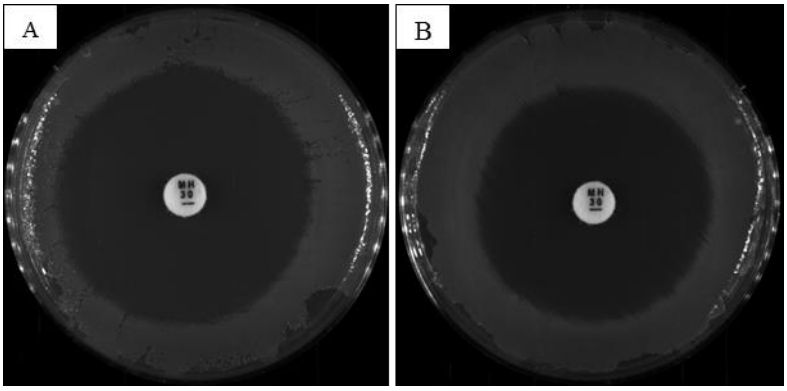


Figure 4.12 Expression in trans of two-component system in K279a *ampR*^{FS} reduces susceptibility to minocycline

A) K279a *ampR*^{FS} carrying pBBR1MCS-4 zone of inhibition 39 mm **B)** K279a *ampR*^{FS} carrying pBBR1MCS-4::two-component system 35 mm

4.3 Conclusions

Regarding aminoglycoside resistance, we found the reason for overexpression of *smeYZ* in *S. maltophilia* that does not involve mutation of the local regulator *smeSyRy.* Here, we found that ribosomal mutations and ribosomal-damaging aminoglycosides activate *smeYZ* overproduction. It may be that this is, like the MexXY situation in *P. aeruginosa*, caused by the accumulation of abnormal polypeptides in the cell. Given the physiological role of *smeYZ (259)* , it is logical to propose that *smeYZ* overproduction offers an advantage in terms of virulence. Although we managed to find an explanation for *smeYZ* overproduction, the cause for *smeDEF* overproduction was more complicated. Apart from loss of function mutations in the local repressor of *smeDEF*, we can only suggest one alternative that involves alteration in a glycosyltransferase, possibly involved in lipid synthesis or the breakdown of a toxic molecule, that act as a signal for constitutive expression of *smeDEF.* Considerable extra work would be needed to identify the signal.

Characterisation of levofloxacin resistant mutants and those with reduced susceptibility to minocycline led us to identify the participation of two novel ABC transporters. Recently the overproduction of the MFS transporter, MfsA, was identified as a quinolone efflux pump in *S. maltophilia* that is also involved in efflux of aminoglycosides, tetracyclines, chloramphenicol, macrolides and cephalosporins (281, 282). SmeVWX an RND transporter has been reported of conferring resistance to

quinolones and tetracyclines when SmeDEF is not being overexproduced. In fact, it has been found that changes at Gly266 in the SmeVWX regulator, repressor SmeRv, are responsible for its overexproduction and consequently quinolone resistance (283). In *S. maltophilia* only two ABC transporters have been identified so far. Whilst SmrA has been associated to quinolone and tetracycline resistance (166), MacABCsm has been linked to aminoglycoside, macrolide and polymixin resistance (181). Here, we identified a bipartite ABC transporter (Smlt2645-6) responsible (in a background that already overproduces SmeDEF) for levofloxacin resistance whose expression is regulated by the local two-component system. Additionally, we identified an ABC transporter (MsbA) possibly involved in lipid export whose overexpression might cause reduced minocycline susceptibility perhaps by altering the hydrophobicity of the outer membrane. Expression of *msbA* might be regulated by a local two component system. However, overexpression of the mutated version of the two-component system had only a minor impact on minocycline susceptibility. In *S. maltophilia,* only one pump has been identified for its role in extrusion of hydrophobic substances and that is EmrCABsm (284) but it has not been linked to tetracycline resistance. This reduced susceptibility to minocycline required two steps and the mutant also overexpresses *smeDEF*. In fact, overexpression of *smeDEF* has been related to reduced susceptibility to tetracycline in the past (168) No common mutations were found to be responsible for upregulation of *smeDEF* in K MIN 2 and the only additional mutation was in a lauroyl acyltransferase (*smlt4167*). It is possible that this enzyme is also involved in detoxifying the cell, and therefore its loss is another way of activating SmeDEF production. Overall, however, minocycline resistance was not seen in mutants – even following multiple steps – or in any of our collection of highly resistant clinical isoaltes. Based on the results of this chapter, it would seem that minocycline, therefore, has a strong future in the clinic for treatment of *S. maltophilia* infections.

4.4 Annex 2

Table 4.6 Normalised proteomics data for KLEV5 relative to KMOX2

Abundance changes significantly according to a t-Test (P<0.05). Proteins that are >1.5 fold up and down regulated are highlighted green or red, respectively. Smaller changes are highlighted in orange.

Accession	Description	T-Test $_{KMOX2\ vs\ KLEV5}$	Fold $_{KMOX2\ vs\ KLEV5}$
B2FHA8	Putative phospholipase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0142 PE=4 SV=1 - [B2FHA8_STRMK]	0.03	0.37
B2FHC0	Putative N-acetylmuramoyl-L-alanine amidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0154 PE=4 SV=1 - [B2FHC0_STRMK]	0.01	0.46
B2FHC6	Superoxide dismutase [Cu-Zn] OS=Stenotrophomonas maltophilia (strain K279a) GN=sodC1 PE=3 SV=1 - [B2FHC6_STRMK]	0.01	0.36
B2FHC7	Superoxide dismutase [Cu-Zn] OS=Stenotrophomonas maltophilia (strain K279a) GN=sodC2 PE=3 SV=1 - [B2FHC7_STRMK]	0.02	0.46
B2FHD0	Putative 3-ketoacyl-CoA thiolase OS=Stenotrophomonas maltophilia (strain K279a) GN=fadI PE=3 SV=1 - [B2FHD0_STRMK]	0.05	0.35
B2FHF3	Putative peroxidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0187 PE=4 SV=1 - [B2FHF3_STRMK]	<0.05	>100
B2FHI7	Glutamate--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=gltX PE=3 SV=1 - [SYE_STRMK]	<0.05	>100
B2FHM1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1476 PE=4 SV=1 - [B2FHM1_STRMK]	0.02	0.45
B2FHN2	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1491 PE=4 SV=1 - [B2FHN2_STRMK]	0.05	0.49
B2FHN5	Lipid-A-disaccharide synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=lpxB PE=3 SV=1 - [B2FHN5_STRMK]	0.02	0.44
B2FHY7	ATP synthase epsilon chain OS=Stenotrophomonas maltophilia (strain K279a) GN=atpC PE=3 SV=1 - [ATPE_STRMK]	0.01	0.25
B2FHY8	ATP synthase subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=atpD PE=3 SV=1 - [ATPB_STRMK]	0.03	0.41
B2FHY9	ATP synthase gamma chain OS=Stenotrophomonas maltophilia (strain K279a) GN=atpG PE=3 SV=1 - [ATPG_STRMK]	0.01	0.35
B2FHZ0	ATP synthase subunit alpha OS=Stenotrophomonas maltophilia (strain K279a) GN=atpA PE=3 SV=1 - [ATPA_STRMK]	0.03	0.38
B2FHZ1	ATP synthase subunit delta OS=Stenotrophomonas maltophilia (strain K279a) GN=atpH PE=3 SV=1 - [ATPD_STRMK]	0.01	0.42
B2FI05	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4128 PE=4 SV=1 - [B2FI05_STRMK]	0.01	0.38
B2FI14	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4137 PE=4 SV=1 - [B2FI14_STRMK]	0.01	0.45
B2FI28	Putative AMP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0208 PE=4 SV=1 - [B2FI28_STRMK]	0.03	0.33
B2FI43	Putative peptidyl-dipeptidase Dcp (Dipeptidyl carboxypeptidase) OS=Stenotrophomonas maltophilia (strain K279a) GN=dcp PE=3 SV=1 - [B2FI43_STRMK]	0.04	0.38
B2FI64	Ribonucleoside-diphosphate reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=RRM1 PE=3 SV=1 - [B2FI64_STRMK]	0.02	3.26
B2FIA0	Outer membrane protein assembly factor BamA OS=Stenotrophomonas maltophilia (strain K279a) GN=bamA PE=3 SV=1 - [B2FIA0_STRMK]	0.05	0.48

B2FIA9	30S ribosomal protein S2 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsB PE=3 SV=1 - [RS2_STRMK]	0.03	0.82
B2FIB6	Methionine aminopeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=map PE=3 SV=1 - [B2FIB6_STRMK]	<0.05	>100
B2FII5	Putative CDP-diacylglycerol pyrophosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2903 PE=4 SV=1 - [B2FII5_STRMK]	0.04	0.51
B2FIJ0	L-lactate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=lldD PE=3 SV=1 - [LLDD_STRMK]	0.03	0.47
B2FIJ2	Putative L-lactate permease OS=Stenotrophomonas maltophilia (strain K279a) GN=lctP PE=4 SV=1 - [B2FIJ2_STRMK]	0.01	0.34
B2FIM7	Putative thiol:disulfide interchange protein OS=Stenotrophomonas maltophilia (strain K279a) GN=dsbE PE=4 SV=1 - [B2FIM7_STRMK]	<0.05	0.00
B2FIQ9	Putative glycosyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4173 PE=4 SV=1 - [B2FIQ9_STRMK]	0.02	0.24
B2FIR9	Putative transmembrane RDD family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4183 PE=4 SV=1 - [B2FIR9_STRMK]	0.01	0.39
B2FIS4	Putative lipid biosynthesis 3-oxoacyl-[acyl-carrier-protein] reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4188 PE=4 SV=1 - [B2FIS4_STRMK]	<0.05	0.00
B2FIT3	Putative pyridine nucleotide-disulphide oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=ndh PE=4 SV=1 - [B2FIT3_STRMK]	0.01	0.36
B2FJ30	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0359 PE=3 SV=1 - [B2FJ30_STRMK]	0.05	0.53
B2FJ75	Putrescine-binding periplasmic protein OS=Stenotrophomonas maltophilia (strain K279a) GN=potF PE=3 SV=1 - [B2FJ75_STRMK]	0.04	0.39
B2FJ81	Putative transmembrane magnesium/cobalt transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=corA PE=4 SV=1 - [B2FJ81_STRMK]	0.02	0.40
B2FJ92	tRNA-2-methylthio-N(6)-dimethylallyladenosine synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=miaB PE=3 SV=1 - [MIAB_STRMK]	<0.05	>100
B2FJC8	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1636 PE=4 SV=1 - [B2FJC8_STRMK]	0.03	0.46
B2FJG9	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2998 PE=4 SV=1 - [B2FJG9_STRMK]	<0.05	0.00
B2FJH7	Putative putative conjugative transfer protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3006 PE=4 SV=1 - [B2FJH7_STRMK]	0.02	0.27
B2FJH9	Putative transmembrane conjugative DNA transfer protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3008 PE=4 SV=1 - [B2FJH9_STRMK]	0.02	0.37
B2FJP7	Putative hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4251 PE=4 SV=1 - [B2FJP7_STRMK]	0.02	0.36
B2FJR1	Putative transmembrane GGDEF signalling protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4266 PE=4 SV=1 - [B2FJR1_STRMK]	0.05	0.52
B2FJR8	Putative isocitrate dehydrogenase [NADP] OS=Stenotrophomonas maltophilia (strain K279a) GN=icd PE=4 SV=1 - [B2FJR8_STRMK]	0.02	0.09
B2FJR9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4275 PE=4 SV=1 - [B2FJR9_STRMK]	0.05	0.27
B2FJT2	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4289 PE=4 SV=1 - [B2FJT2_STRMK]	0.04	0.31
B2FJU3	30S ribosomal protein S9 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsI PE=3 SV=1 - [RS9_STRMK]	0.01	0.44
B2FJU4	50S ribosomal protein L13 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplM PE=3 SV=1 - [RL13_STRMK]	0.02	1.77
B2FJW5	Putative phosphomannomutase OS=Stenotrophomonas maltophilia (strain K279a) GN=spgM PE=4 SV=1 - [B2FJW5_STRMK]	0.04	0.21
B2FJX2	Orotate phosphoribosyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=pyrE PE=3 SV=1 - [PYRE_STRMK]	<0.05	>100
B2FJY1	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0420 PE=4 SV=1 - [B2FJY1_STRMK]	0.03	0.34

B2FJY4	Putative fatty acid transport system, membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0423 PE=4 SV=1 - [B2FJY4_STRMK]	0.01	0.34
B2FK09	Putative mechanosensitive ion channel OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0448 PE=4 SV=1 - [B2FK09_STRMK]	0.05	0.46
B2FK29	Putative outer membrane efflux protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1651 PE=4 SV=1 - [B2FK29_STRMK]	0.00	0.08
B2FK30	Putative ABC transport system, membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1652 PE=4 SV=1 - [B2FK30_STRMK]	<0.05	>100
B2FK31	Putative ABC transporter ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1653 PE=3 SV=1 - [B2FK31_STRMK]	<0.05	>100
B2FK32	Putative HlyD family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1654 PE=4 SV=1 - [B2FK32_STRMK]	<0.05	>100
B2FK71	Peptidyl-prolyl cis-trans isomerase OS=Stenotrophomonas maltophilia (strain K279a) GN=fkbP PE=4 SV=1 - [B2FK71_STRMK]	0.04	0.51
B2FK77	Putative transmembrane CorC/HlyC family transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1704 PE=4 SV=1 - [B2FK77_STRMK]	0.04	0.42
B2FK84	CTP synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=pyrG PE=3 SV=1 - [PYRG_STRMK]	<0.05	>100
B2FK97	Putative subfamily M23B unassigned peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1724 PE=4 SV=1 - [B2FK97_STRMK]	0.03	0.39
B2FKA2	ATP-dependent zinc metalloprotease FtsH OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsH PE=3 SV=1 - [B2FKA2_STRMK]	0.04	0.42
B2FKF1	Conserved hypothetical FHA domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3099 PE=4 SV=1 - [B2FKF1_STRMK]	0.02	0.42
B2FKJ3	Cell division protein ZipA OS=Stenotrophomonas maltophilia (strain K279a) GN=zipA PE=3 SV=1 - [B2FKJ3_STRMK]	0.04	0.40
B2FKL0	Putative haloacid dehalogenase hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4308 PE=4 SV=1 - [B2FKL0_STRMK]	0.03	0.49
B2FKN5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4334 PE=4 SV=1 - [B2FKN5_STRMK]	0.04	0.24
B2FKN6	Putative peptide transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4335 PE=3 SV=1 - [B2FKN6_STRMK]	0.02	0.28
B2FKP6	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4346 PE=4 SV=1 - [B2FKP6_STRMK]	0.01	0.43
B2FKR0	Biosynthetic arginine decarboxylase OS=Stenotrophomonas maltophilia (strain K279a) GN=speA PE=3 SV=1 - [B2FKR0_STRMK]	<0.05	>100
B2FKV5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0481 PE=4 SV=1 - [B2FKV5_STRMK]	<0.05	0.00
B2FL00	Putative NHL repeat protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0528 PE=4 SV=1 - [B2FL00_STRMK]	0.01	0.33
B2FL33	Alanine--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=alaS PE=3 SV=1 - [SYA_STRMK]	<0.05	>100
B2FL57	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1769 PE=4 SV=1 - [B2FL57_STRMK]	0.04	0.20
B2FL62	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1774 PE=4 SV=1 - [B2FL62_STRMK]	0.01	0.38
B2FL73	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1785 PE=4 SV=1 - [B2FL73_STRMK]	0.01	0.40
B2FL82	Putative ABC transporter ATP-binding subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1794 PE=4 SV=1 - [B2FL82_STRMK]	0.04	0.42
B2FLC2	Putative ABC transporter, ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3186 PE=4 SV=1 - [B2FLC2_STRMK]	0.04	0.52
B2FLC6	Conserved hypothetical exported protein with low similarity to flagellar FliL protein OS=Stenotrophomonas maltophilia (strain	0.03	0.37

	K279a) GN=Smlt3190 PE=4 SV=1 - [B2FLC6_STRMK]		
B2FLD2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex OS=Stenotrophomonas maltophilia (strain K279a) GN=sucB PE=3 SV=1 - [B2FLD2_STRMK]	0.04	3.31
B2FLF2	Putative phospholipase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3218 PE=4 SV=1 - [B2FLF2_STRMK]	0.03	0.43
B2FLG5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3232 PE=4 SV=1 - [B2FLG5_STRMK]	0.03	0.37
B2FLG9	Putative peptide transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3236 PE=3 SV=1 - [B2FLG9_STRMK]	0.02	0.52
B2FLN0	Putative pyridoxal-phosphate dependent enzyme OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4470 PE=4 SV=1 - [B2FLN0_STRMK]	<0.05	>100
B2FLT1	Asparagine synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0598 PE=4 SV=1 - [B2FLT1_STRMK]	<0.05	>100
B2FLU9	Putative ABC transporter component, polysaccharide related OS=Stenotrophomonas maltophilia (strain K279a) GN=wzt PE=4 SV=1 - [B2FLU9_STRMK]	0.04	0.38
B2FLV2	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=wxocA PE=4 SV=1 - [B2FLV2_STRMK]	0.02	0.45
B2FLV6	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0626 PE=4 SV=1 - [B2FLV6_STRMK]	0.03	0.51
B2FLW2	Chaperone protein HtpG OS=Stenotrophomonas maltophilia (strain K279a) GN=htpG PE=3 SV=1 - [B2FLW2_STRMK]	0.03	4.85
B2FLW5	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1812 PE=4 SV=1 - [B2FLW5_STRMK]	0.04	0.38
B2FLW7	Putative gamma-glutamyltranspeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=ggt PE=4 SV=1 - [B2FLW7_STRMK]	<0.05	>100
B2FM92	Ribonuclease E OS=Stenotrophomonas maltophilia (strain K279a) GN=rnE PE=3 SV=1 - [B2FM92_STRMK]	0.02	2.12
B2FM97	Putative substrate-binding component of ABC transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3252 PE=4 SV=1 - [B2FM97_STRMK]	0.01	0.39
B2FMB8	Putative cytochrome C-type biogenesis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=dsbE PE=4 SV=1 - [B2FMB8_STRMK]	0.02	0.19
B2FMI1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4498 PE=4 SV=1 - [B2FMI1_STRMK]	0.00	0.41
B2FMI8	UPF0114 protein Smlt4505 OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4505 PE=3 SV=1 - [B2FMI8_STRMK]	0.05	0.68
B2FMI9	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4506 PE=4 SV=1 - [B2FMI9_STRMK]	0.01	0.23
B2FMJ3	Putative ABC transmembrane permease component transport system protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4510 PE=4 SV=1 - [B2FMJ3_STRMK]	0.00	0.29
B2FMK1	Putative ABC transporter ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4518 PE=3 SV=1 - [B2FMK1_STRMK]	0.04	0.46
B2FML3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4532 PE=4 SV=1 - [B2FML3_STRMK]	0.05	0.14
B2FMN4	Putative FAD-binding oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0633 PE=4 SV=1 - [B2FMN4_STRMK]	0.04	0.37
B2FMN5	Putative transmembrane UbiA prenyltransferase family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0634 PE=4 SV=1 - [B2FMN5_STRMK]	0.03	0.39
B2FMN6	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0635 PE=4 SV=1 - [B2FMN6_STRMK]	0.03	0.50
B2FMP5	Putative electron transfer flavoprotein subunit alpha OS=Stenotrophomonas maltophilia (strain K279a) GN=etfA PE=4 SV=1 - [B2FMP5_STRMK]	<0.05	>100
B2FMQ7	Putative glycosyltransferase protein OS=Stenotrophomonas maltophilia (strain K279a) GN=wxocA PE=4 SV=1 - [B2FMQ7_STRMK]	0.05	0.43

B2FMR0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0660 PE=4 SV=1 - [B2FMR0_STRMK]	0.05	0.41
B2FMR2	Putative permease component of ABC transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0662 PE=4 SV=1 - [B2FMR2_STRMK]	0.03	0.53
B2FMS6	Putative transmembrane permease protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0677 PE=4 SV=1 - [B2FMS6_STRMK]	0.02	0.31
B2FN03	Protein-export membrane protein SecF OS=Stenotrophomonas maltophilia (strain K279a) GN=secF PE=3 SV=1 - [B2FN03_STRMK]	0.02	0.55
B2FN30	Protease HtpX OS=Stenotrophomonas maltophilia (strain K279a) GN=htpX PE=3 SV=1 - [HTPX_STRMK]	0.01	0.55
B2FN37	Putative N-acetylmuramoyl-L-alanine amidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3330 PE=4 SV=1 - [B2FN37_STRMK]	0.02	0.46
B2FN90	Translation initiation factor IF-2 OS=Stenotrophomonas maltophilia (strain K279a) GN=infB PE=3 SV=1 - [IF2_STRMK]	0.05	4.23
B2FN93	NADH-quinone oxidoreductase subunit N OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoN PE=3 SV=1 - [B2FN93_STRMK]	0.02	0.46
B2FN94	Putative NADH dehydrogenase I chain M OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoM PE=4 SV=1 - [B2FN94_STRMK]	0.05	0.65
B2FNC9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4585 PE=4 SV=1 - [B2FNC9_STRMK]	<0.05	>100
B2FND7	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4593 PE=4 SV=1 - [B2FND7_STRMK]	0.00	0.29
B2FNG3	Aldehyde dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=calB PE=3 SV=1 - [B2FNG3_STRMK]	<0.05	0.00
B2FNL4	N utilization substance protein B homolog OS=Stenotrophomonas maltophilia (strain K279a) GN=nusB PE=3 SV=1 - [NUSB_STRMK]	<0.05	>100
B2FNN2	Putative penicillin-binding protein 3 OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsI PE=4 SV=1 - [B2FNN2_STRMK]	0.05	0.50
B2FNN7	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=murG PE=3 SV=1 - [B2FNN7_STRMK]	0.03	0.35
B2FNP5	30S ribosomal protein S1 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsA PE=3 SV=1 - [B2FNP5_STRMK]	0.04	2.52
B2FNR0	Outer membrane protein assembly factor BamB OS=Stenotrophomonas maltophilia (strain K279a) GN=bamB PE=3 SV=1 - [B2FNR0_STRMK]	0.04	0.36
B2FNX3	Putative NADH-ubiquinone oxidoreductase, 75 kDa subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoG PE=4 SV=1 - [B2FNX3_STRMK]	0.03	0.44
B2FNX4	Putative NADH dehydrogenase I chain F OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoF PE=4 SV=1 - [B2FNX4_STRMK]	0.02	0.29
B2FNX6	NADH-quinone oxidoreductase subunit D OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoD PE=3 SV=1 - [NUOD_STRMK]	0.04	0.52
B2FNY0	Putative general secretory pathway protein-export membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=secG PE=4 SV=1 - [B2FNY0_STRMK]	0.03	0.43
B2FNY5	Putative lipopolysaccharide core oligosaccharide biosynthesis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3412 PE=4 SV=1 - [B2FNY5_STRMK]	0.02	0.42
B2FP04	Phosphatidylserine decarboxylase proenzyme OS=Stenotrophomonas maltophilia (strain K279a) GN=psd PE=3 SV=1 - [PSD_STRMK]	0.04	0.48
B2FP06	Putative membrane-bound lytic murein transglycosylase d OS=Stenotrophomonas maltophilia (strain K279a) GN=mltD PE=4 SV=1 - [B2FP06_STRMK]	0.03	0.36
B2FP17	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3446 PE=3 SV=1 - [B2FP17_STRMK]	0.00	0.49
B2FP19	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3449 PE=4 SV=1 - [B2FP19_STRMK]	0.00	0.27

B2FP30	Putative lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3460 PE=4 SV=1 - [B2FP30_STRMK]	0.04	0.28
B2FP33	Putative alkaline phosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3463 PE=3 SV=1 - [B2FP33_STRMK]	0.04	0.48
B2FP55	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4642 PE=4 SV=1 - [B2FP55_STRMK]	<0.05	>100
B2FP83	Putative transmembrane component of ABC transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4671 PE=4 SV=1 - [B2FP83_STRMK]	0.01	0.34
B2FP97	Putative amino-acid transporter transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4685 PE=4 SV=1 - [B2FP97_STRMK]	0.00	0.29
B2FPD2	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0785 PE=4 SV=1 - [B2FPD2_STRMK]	0.01	0.78
B2FPE9	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0802 PE=4 SV=1 - [B2FPE9_STRMK]	<0.05	0.00
B2FPF2	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0805 PE=4 SV=1 - [B2FPF2_STRMK]	0.00	0.55
B2FPH3	Putative 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase OS=Stenotrophomonas maltophilia (strain K279a) GN=ubiF PE=4 SV=1 - [B2FPH3_STRMK]	0.03	0.38
B2FPK0	Putative repetitive protein with two-component sensor and regulator motifs OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2141 PE=4 SV=1 - [B2FPK0_STRMK]	0.02	0.28
B2FPN1	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2175 PE=3 SV=1 - [B2FPN1_STRMK]	<0.05	0.00
B2FPN2	Putative copper-transporting P-type ATPase OS=Stenotrophomonas maltophilia (strain K279a) GN=actP PE=3 SV=1 - [B2FPN2_STRMK]	0.01	0.37
B2FPQ4	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2199 PE=4 SV=1 - [B2FPQ4_STRMK]	<0.05	0.00
B2FPR0	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3478 PE=3 SV=1 - [B2FPR0_STRMK]	0.04	0.47
B2FPR6	LPS-assembly lipoprotein LptE OS=Stenotrophomonas maltophilia (strain K279a) GN=lptE PE=3 SV=1 - [B2FPR6_STRMK]	0.03	0.45
B2FPR7	Leucine--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=leuS PE=3 SV=1 - [SYL_STRMK]	<0.05	>100
B2FPS1	Putative transmembrane GGDEF transcriptional regulatory protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3489 PE=4 SV=1 - [B2FPS1_STRMK]	0.05	0.88
B2FPV5	Putative autotransporter subtilisin-like protease OS=Stenotrophomonas maltophilia (strain K279a) GN=sphB PE=4 SV=1 - [B2FPV5_STRMK]	<0.05	0.00
B2FPY0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3550 PE=4 SV=1 - [B2FPY0_STRMK]	0.01	0.28
B2FQ16	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0872 PE=4 SV=1 - [B2FQ16_STRMK]	<0.05	>100
B2FQ28	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0885 PE=3 SV=1 - [B2FQ28_STRMK]	0.04	0.39
B2FQ31	Elongation factor Tu OS=Stenotrophomonas maltophilia (strain K279a) GN=tufB PE=3 SV=1 - [B2FQ31_STRMK]	0.02	3.80
B2FQ36	50S ribosomal protein L10 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplJ PE=3 SV=1 - [RL10_STRMK]	0.04	1.99
B2FQ39	DNA-directed RNA polymerase subunit beta' OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoC PE=3 SV=1 - [RPOC_STRMK]	0.04	7.85
B2FQ42	Elongation factor G OS=Stenotrophomonas maltophilia (strain K279a) GN=fusA PE=3 SV=1 - [EFG_STRMK]	0.04	4.24
B2FQ48	50S ribosomal protein L2 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplB PE=3 SV=1 - [RL2_STRMK]	0.03	0.64
B2FQ55	Putative multidrug ACR family efflux system OS=Stenotrophomonas maltophilia (strain K279a) GN=smeZ PE=4 SV=1 - [B2FQ55_STRMK]	0.01	8.78
B2FQ64	Putative ferrous iron transport protein B OS=Stenotrophomonas maltophilia (strain K279a) GN=feoB PE=4 SV=1 - [B2FQ64_STRMK]	0.03	0.57

B2FQC4	Elongation factor 4 OS=Stenotrophomonas maltophilia (strain K279a) GN=lepA PE=3 SV=1 - [LEPA_STRMK]	0.05	3.22
B2FQE1	Putative transmembrane sulfatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3571 PE=4 SV=1 - [B2FQE1_STRMK]	0.02	0.35
B2FQE3	Putative angiotensin-converting enzyme like peptidyl dipeptidase protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3574 PE=4 SV=1 - [B2FQE3_STRMK]	0.05	0.47
B2FQH1	Putative endopeptidase inhibitor (Alpha-2-macroglobulin like protein) OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3603 PE=4 SV=1 - [B2FQH1_STRMK]	0.02	0.27
B2FQK5	30S ribosomal protein S13 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsM PE=3 SV=1 - [RS13_STRMK]	0.05	1.30
B2FQK6	30S ribosomal protein S11 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsK PE=3 SV=1 - [RS11_STRMK]	0.01	0.79
B2FQK8	DNA-directed RNA polymerase subunit alpha OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoA PE=3 SV=1 - [RPOA_STRMK]	0.03	4.78
B2FQL8	Malate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=mdh PE=3 SV=1 - [MDH_STRMK]	<0.05	>100
B2FQN1	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0958 PE=4 SV=1 - [B2FQN1_STRMK]	<0.05	0.00
B2FQN3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0960 PE=4 SV=1 - [B2FQN3_STRMK]	0.00	0.34
B2FQP8	Putative exopolyphosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=ppx PE=4 SV=1 - [B2FQP8_STRMK]	<0.05	>100
B2FQR4	Lon protease OS=Stenotrophomonas maltophilia (strain K279a) GN=lon PE=2 SV=1 - [B2FQR4_STRMK]	<0.05	>100
B2FQR5	Putative DNA-binding protein HU-beta OS=Stenotrophomonas maltophilia (strain K279a) GN=hupB PE=3 SV=1 - [B2FQR5_STRMK]	<0.05	>100
B2FQY4	Putative cell division protein OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsK PE=4 SV=1 - [B2FQY4_STRMK]	0.04	0.48
B2FR06	33 kDa chaperonin OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3643 PE=4 SV=1 - [B2FR06_STRMK]	<0.05	>100
B2FR08	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3645 PE=3 SV=1 - [B2FR08_STRMK]	0.00	0.26
B2FR11	Putative acyl-coenzyme A dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=fadE PE=4 SV=1 - [B2FR11_STRMK]	0.05	0.49
B2FR15	Putative beta-lactamase protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3652 PE=4 SV=1 - [B2FR15_STRMK]	0.02	0.37
B2FR23	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3660 PE=4 SV=1 - [B2FR23_STRMK]	0.01	0.23
B2FR62	Putative autotransporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1001 PE=4 SV=1 - [B2FR62_STRMK]	0.02	0.49
B2FR78	Putative outer membrane lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1018 PE=4 SV=1 - [B2FR78_STRMK]	0.05	0.30
B2FRM8	Putative peptidoglycan-associated lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3703 PE=3 SV=1 - [B2FRM8_STRMK]	0.02	0.43
B2FRM9	Protein TolB OS=Stenotrophomonas maltophilia (strain K279a) GN=tolB PE=3 SV=1 - [B2FRM9_STRMK]	0.04	0.41
B2FRP3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3720 PE=4 SV=1 - [B2FRP3_STRMK]	0.02	0.24
B2FRP4	Putative transmembrane Na+/H+ antiporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3721 PE=4 SV=1 - [B2FRP4_STRMK]	0.04	0.45
B2FRP8	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3725 PE=3 SV=1 - [B2FRP8_STRMK]	0.03	0.42
B2FRP9	Putative transmembrane transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3726 PE=4 SV=1 - [B2FRP9_STRMK]	0.03	0.40
B2FRQ2	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3730 PE=4 SV=1 - [B2FRQ2_STRMK]	0.00	0.24
B2FRR0	Putative lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3739 PE=4 SV=1 - [B2FRR0_STRMK]	0.01	0.37
B2FRS8	Putative class II pilin PilE OS=Stenotrophomonas maltophilia (strain K279a) GN=pilE PE=3 SV=1 - [B2FRS8_STRMK]	0.01	0.43

B2FRS9	Putative pilus-assembly protein OS=Stenotrophomonas maltophilia (strain K279a) GN=pilG PE=3 SV=1 - [B2FRS9_STRMK]	<0.05	0
B2FRV6	Putative MgtE/divalent cation transmembrane transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1103 PE=4 SV=1 - [B2FRV6_STRMK]	0.02	0.38
B2FRX9	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1127 PE=4 SV=1 - [B2FRX9_STRMK]	0.03	0.40
B2FRZ0	Putative oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1138 PE=4 SV=1 - [B2FRZ0_STRMK]	0.05	0.42
B2FRZ4	Putative transmembrane PepSY family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1142 PE=4 SV=1 - [B2FRZ4_STRMK]	0.03	0.55
B2FS06	Putative ABC transporter, ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1155 PE=4 SV=1 - [B2FS06_STRMK]	<0.05	>100
B2FS41	Putative cobalt-zinc-cadmium resistance protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2459 PE=4 SV=1 - [B2FS41_STRMK]	<0.05	0.00
B2FSD7	Large-conductance mechanosensitive channel OS=Stenotrophomonas maltophilia (strain K279a) GN=mscL PE=3 SV=1 - [MSCL_STRMK]	0.05	0.43
B2FSE0	Putative transmembrane symporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3785 PE=4 SV=1 - [B2FSE0_STRMK]	0.03	0.23
B2FSE3	Putative HlyD-family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeM PE=4 SV=1 - [B2FSE3_STRMK]	0.02	0.55
B2FSF0	Fructose-bisphosphate aldolase OS=Stenotrophomonas maltophilia (strain K279a) GN=alf1 PE=3 SV=1 - [B2FSF0_STRMK]	0.03	1.95
B2FSG6	Putative transmembrane sodium-dicarboxylate family transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3814 PE=4 SV=1 - [B2FSG6_STRMK]	0.01	0.43
B2FSH3	Putative type II/III pilus secretin OS=Stenotrophomonas maltophilia (strain K279a) GN=pilQ PE=4 SV=1 - [B2FSH3_STRMK]	<0.05	0
B2FSH5	Putative PilO protein (Type 4 fimbrial biogenesis protein PilO) OS=Stenotrophomonas maltophilia (strain K279a) GN=pilO PE=4 SV=1 - [B2FSH5_STRMK]	0.01	0.12
B2FSJ9	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1175 PE=4 SV=1 - [B2FSJ9_STRMK]	0.00	0.35
B2FSN4	Putative transmembrane phosphoesterase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1210 PE=4 SV=1 - [B2FSN4_STRMK]	0.02	0.30
B2FSS6	Putative PTS system, fructose-specific IIBC component OS=Stenotrophomonas maltophilia (strain K279a) GN=fruA PE=4 SV=1 - [B2FSS6_STRMK]	0.01	0.48
B2FSS7	Putative outer membrane regulator of pathogenicity factors protein OS=Stenotrophomonas maltophilia (strain K279a) GN=rpfN PE=4 SV=1 - [B2FSS7_STRMK]	0.00	0.39
B2FT31	Putative ACR family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3869 PE=4 SV=1 - [B2FT31_STRMK]	0.03	0.38
B2FT66	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3905 PE=3 SV=1 - [B2FT66_STRMK]	0.02	0.18
B2FT68	Putative transcriptional regulator OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3907 PE=4 SV=1 - [B2FT68_STRMK]	0.04	0.42
B2FT83	DNA gyrase subunit B OS=Stenotrophomonas maltophilia (strain K279a) GN=gyrB PE=3 SV=1 - [B2FT83_STRMK]	0.04	5.01
B2FTA2	Putative oligopeptide transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1245 PE=4 SV=1 - [B2FTA2_STRMK]	0.05	0.38
B2FTA8	Probable septum site-determining protein MinC OS=Stenotrophomonas maltophilia (strain K279a) GN=minC PE=3 SV=1 - [B2FTA8_STRMK]	<0.05	>100
B2FTJ7	**Macrolide export ATP-binding/permease protein MacB OS=Stenotrophomonas maltophilia (strain K279a) GN=macB PE=3 SV=1 - [B2FTJ7_STRMK]**	<0.05	>100
B2FTJ8	**Putative HlyD family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2643 PE=4 SV=1 -**	<0.05	>100

	[B2FTJ8_STRMK]		
B2FTK0	**Putative two-component regulatory system family, response regulator protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2645 PE=4 SV=1 - [B2FTK0_STRMK]**	<0.05	>100
B2FTK1	**Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2646 PE=4 SV=1 - [B2FTK1_STRMK]**	0.01	9.60
B2FTS7	Putative 3-deoxy-D-manno-octulosonic-acid transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=kdtA PE=4 SV=1 - [B2FTS7_STRMK]	0.05	0.38
B2FTS8	Putative lipid A biosynthesis lauroyl acyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=htrB PE=4 SV=1 - [B2FTS8_STRMK]	0.03	0.36
B2FU10	Fructose-1,6-bisphosphatase class 1 OS=Stenotrophomonas maltophilia (strain K279a) GN=fbp PE=3 SV=1 - [F16PA_STRMK]	<0.05	>100
B2FU42	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0083 PE=3 SV=1 - [B2FU42_STRMK]	0.04	0.53
B2FU43	Acid phosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0084 PE=3 SV=1 - [B2FU43_STRMK]	0.02	0.35
B2FU94	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1354 PE=4 SV=1 - [B2FU94_STRMK]	0.05	0.25
B2FUA1	Putative quinol oxidase subunit 1 OS=Stenotrophomonas maltophilia (strain K279a) GN=qoxB PE=3 SV=1 - [B2FUA1_STRMK]	0.01	0.38
B2FUA6	Putative chaperone protein HtpG (Heat shock protein HtpG) OS=Stenotrophomonas maltophilia (strain K279a) GN=htpG PE=4 SV=1 - [B2FUA6_STRMK]	<0.05	>100
B2FUB6	50S ribosomal protein L19 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplS PE=3 SV=1 - [RL19_STRMK]	0.02	0.67
B2FUE6	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1413 PE=4 SV=1 - [B2FUE6_STRMK]	<0.05	>100
B2FUE7	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1414 PE=4 SV=1 - [B2FUE7_STRMK]	<0.05	>100
B2FUF0	Putative nucleotide sugar transaminase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1417 PE=3 SV=1 - [B2FUF0_STRMK]	<0.05	>100
B2FUL3	Putative Major Facilitator Superfamily transmembrane nitrite extrusion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=narK PE=4 SV=1 - [B2FUL3_STRMK]	0.05	0.40
B2FUR1	Putative TonB-dependent outer membrane receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4026 PE=3 SV=1 - [B2FUR1_STRMK]	0.02	0.42
B2FUT1	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4049 PE=4 SV=1 - [B2FUT1_STRMK]	0.05	0.43
B2FUT3	Putative exported rare lipoprotein A OS=Stenotrophomonas maltophilia (strain K279a) GN=rlpA PE=3 SV=1 - [B2FUT3_STRMK]	0.04	0.30
B2FUT6	Putative rod shape-determining protein RodA OS=Stenotrophomonas maltophilia (strain K279a) GN=mrdB PE=4 SV=1 - [B2FUT6_STRMK]	<0.05	0

4.5 Annex 3

Table 4.7 Normalised proteomics data for KMIN2 relative to K279a

Abundance changes significantly according to a t-Test (P<0.05). Proteins that are >1.5 fold up and down regulated are highlighted green or red, respectively. Smaller changes are highlighted in orange.

Accession	Description	T-Test $_{K279a\ vs\ KMIN2}$	Fold $_{K279a\ vs\ KMIN2}$
B2FHB0	Putative transmembrane acetyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0144 PE=4 SV=1 - [B2FHB0_STRMK]	0.01	1.48
B2FHC0	Putative N-acetylmuramoyl-L-alanine amidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0154 PE=4 SV=1 - [B2FHC0_STRMK]	0.02	0.78
B2FHD1	Putative transmembrane HemY porphyrin biosynthesis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0165 PE=4 SV=1 - [B2FHD1_STRMK]	0.03	1.20
B2FHF0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0184 PE=4 SV=1 - [B2FHF0_STRMK]	0.00	3.12
B2FHG4	Putative electron transfer flavoprotein-ubiquinone oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0198 PE=4 SV=1 - [B2FHG4_STRMK]	0.04	1.35
B2FHG9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0204 PE=4 SV=1 - [B2FHG9_STRMK]	0.03	1.30
B2FHH2	Putative TonB dependent siderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1426 PE=3 SV=1 - [B2FHH2_STRMK]	0.03	1.31
B2FHI0	Putative acyl-CoA synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1434 PE=4 SV=1 - [B2FHI0_STRMK]	0.01	2.49
B2FHJ2	Putative outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1446 PE=3 SV=1 - [B2FHJ2_STRMK]	0.02	0.77
B2FHL6	Putative ABC transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1471 PE=3 SV=1 - [B2FHL6_STRMK]	0.01	1.68
B2FHN5	Lipid-A-disaccharide synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=lpxB PE=3 SV=1 - [B2FHN5_STRMK]	0.02	0.67
B2FHP7	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2807 PE=4 SV=1 - [B2FHP7_STRMK]	0.04	1.65
B2FHR8	Putative autotransporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2834 PE=4 SV=1 - [B2FHR8_STRMK]	0.01	0.53
B2FHR9	Putative TonB-dependent outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2835 PE=3 SV=1 - [B2FHR9_STRMK]	0.03	1.27
B2FHT9	Putative iron transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2858 PE=3 SV=1 - [B2FHT9_STRMK]	0.01	1.60
B2FHY3	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4106 PE=4 SV=1 - [B2FHY3_STRMK]	0.04	1.25
B2FHY9	ATP synthase gamma chain OS=Stenotrophomonas maltophilia (strain K279a) GN=atpG PE=3 SV=1 - [ATPG_STRMK]	0.05	0.47
B2FHZ0	ATP synthase subunit alpha OS=Stenotrophomonas maltophilia (strain K279a) GN=atpA PE=3 SV=1 - [ATPA_STRMK]	0.01	0.46

B2FHZ1	ATP synthase subunit delta OS=Stenotrophomonas maltophilia (strain K279a) GN=atpH PE=3 SV=1 - [ATPD_STRMK]	0.02	0.37
B2FHZ2	ATP synthase subunit b OS=Stenotrophomonas maltophilia (strain K279a) GN=atpF PE=3 SV=1 - [ATPF_STRMK]	0.04	1.23
B2FHZ4	ATP synthase subunit a OS=Stenotrophomonas maltophilia (strain K279a) GN=atpB PE=3 SV=1 - [B2FHZ4_STRMK]	0.01	1.80
B2FHZ8	Acetyltransferase component of pyruvate dehydrogenase complex OS=Stenotrophomonas maltophilia (strain K279a) GN=pdhB PE=3 SV=1 - [B2FHZ8_STRMK]	0.00	2.22
B2FI05	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4128 PE=4 SV=1 - [B2FI05_STRMK]	0.02	0.75
B2FI12	Putative colicin I receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=cirA PE=3 SV=1 - [B2FI12_STRMK]	0.04	0.62
B2FI22	Putative extracellular serine protease OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4145 PE=4 SV=1 - [B2FI22_STRMK]	0.05	0.79
B2FI29	Putative NAD(P)H dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0209 PE=4 SV=1 - [B2FI29_STRMK]	0.01	0.35
B2FI32	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0212 PE=4 SV=1 - [B2FI32_STRMK]	0.01	1.53
B2FI33	Probable protein kinase UbiB OS=Stenotrophomonas maltophilia (strain K279a) GN=ubiB PE=4 SV=1 - [B2FI33_STRMK]	0.02	1.45
B2FI94	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0278 PE=4 SV=1 - [B2FI94_STRMK]	0.00	1.81
B2FIA1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1499 PE=4 SV=1 - [B2FIA1_STRMK]	0.00	3.97
B2FIA3	Phosphatidate cytidylyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=cdsA PE=3 SV=1 - [B2FIA3_STRMK]	0.01	1.70
B2FIA9	30S ribosomal protein S2 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsB PE=3 SV=1 - [RS2_STRMK]	0.00	1.62
B2FIC3	Putative penicillin acylase 2 OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1522 PE=4 SV=1 - [B2FIC3_STRMK]	0.01	1.68
B2FIC5	DNA topoisomerase 4 subunit A OS=Stenotrophomonas maltophilia (strain K279a) GN=parC PE=3 SV=1 - [B2FIC5_STRMK]	0.02	1.37
B2FID6	23S rRNA (guanosine-2'-O-)-methyltransferase RlmB OS=Stenotrophomonas maltophilia (strain K279a) GN=rlmB PE=3 SV=1 - [B2FID6_STRMK]	0.04	1.95
B2FIF4	Putative polyphosphate-selective porin O OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1555 PE=4 SV=1 - [B2FIF4_STRMK]	<0.05	>100
B2FIJ2	Putative L-lactate permease OS=Stenotrophomonas maltophilia (strain K279a) GN=lctP PE=4 SV=1 - [B2FIJ2_STRMK]	0.03	1.24
B2FIN7	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4151 PE=3 SV=1 - [B2FIN7_STRMK]	>0.05	0.00
B2FIQ3	Putative lipid A biosynthesis lauroyl acyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=htrB PE=4 SV=1 - [B2FIQ3_STRMK]	0.00	0.25
B2FIQ9	Putative glycosyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4173 PE=4 SV=1 - [B2FIQ9_STRMK]	0.01	0.30
B2FIR8	Putative fimbrial protein (Pilin) OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4182 PE=4 SV=1 - [B2FIR8_STRMK]	0.02	1.38
B2FIR9	Putative transmembrane RDD family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4183 PE=4 SV=1 - [B2FIR9_STRMK]	0.00	0.59
B2FIS7	Putative drug resistance transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4191 PE=4 SV=1 - [B2FIS7_STRMK]	0.04	1.41
B2FIS9	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4193 PE=4 SV=1 - [B2FIS9_STRMK]	0.00	10.80

B2FIU9	60 kDa chaperonin OS=Stenotrophomonas maltophilia (strain K279a) GN=groL PE=3 SV=1 - [CH60_STRMK]	0.01	2.44
B2FJ52	Putative protease OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0381 PE=4 SV=1 - [B2FJ52_STRMK]	0.02	2.02
B2FJB1	Putative oar family adhesion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1619 PE=4 SV=1 - [B2FJB1_STRMK]	0.03	0.74
B2FJB8	Putative PilE protein (Type 4 fimbrial biogenesis protein PilE) OS=Stenotrophomonas maltophilia (strain K279a) GN=pilE PE=4 SV=1 - [B2FJB8_STRMK]	0.05	0.61
B2FJC8	**Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1636 PE=4 SV=1 - [B2FJC8_STRMK]**	**0.00**	**2.07**
B2FJG0	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2989 PE=4 SV=1 - [B2FJG0_STRMK]	0.02	0.43
B2FJH5	Putative putative bacterial secretion system protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3004 PE=4 SV=1 - [B2FJH5_STRMK]	0.01	0.39
B2FJH6	Putative VirB9 OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3005 PE=4 SV=1 - [B2FJH6_STRMK]	0.02	0.32
B2FJH9	Putative transmembrane conjugative DNA transfer protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3008 PE=4 SV=1 - [B2FJH9_STRMK]	0.01	0.50
B2FJJ3	Putative hydroxamate-type ferrisiderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3022 PE=3 SV=1 - [B2FJJ3_STRMK]	0.01	1.83
B2FJQ7	Putative cell division ATP-binding protein FtsE OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsE PE=3 SV=1 - [B2FJQ7_STRMK]	0.02	0.51
B2FJR8	Putative isocitrate dehydrogenase [NADP] OS=Stenotrophomonas maltophilia (strain K279a) GN=icd PE=4 SV=1 - [B2FJR8_STRMK]	0.01	2.92
B2FJR9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4275 PE=4 SV=1 - [B2FJR9_STRMK]	>0.05	0.00
B2FJS2	Putative amidohydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4278 PE=4 SV=1 - [B2FJS2_STRMK]	<0.05	>100
B2FJS4	Putative multidrug efflux system transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4280 PE=4 SV=1 - [B2FJS4_STRMK]	0.04	1.61
B2FJS8	Putative Major Facilitator Superfamily transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4284 PE=4 SV=1 - [B2FJS8_STRMK]	0.01	2.16
B2FJU4	50S ribosomal protein L13 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplM PE=3 SV=1 - [RL13_STRMK]	0.02	1.41
B2FJV0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0387 PE=4 SV=1 - [B2FJV0_STRMK]	0.00	1.53
B2FJV9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0397 PE=4 SV=1 - [B2FJV9_STRMK]	0.03	1.31
B2FJX1	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0410 PE=4 SV=1 - [B2FJX1_STRMK]	0.05	0.61
B2FJX4	Putative beta-lactamase induction signal transducer AmpG OS=Stenotrophomonas maltophilia (strain K279a) GN=ampG PE=4 SV=1 - [B2FJX4_STRMK]	<0.05	>100
B2FJX9	Putative aminopeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0418 PE=4 SV=1 - [B2FJX9_STRMK]	0.05	1.30
B2FJY1	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0420 PE=4 SV=1 - [B2FJY1_STRMK]	0.01	1.45
B2FJY4	Putative fatty acid transport system, membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0423 PE=4 SV=1 - [B2FJY4_STRMK]	0.00	0.45
B2FK09	Putative mechanosensitive ion channel OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0448 PE=4 SV=1 - [B2FK09_STRMK]	0.00	1.44

B2FK21	**Lipid A export ATP-binding/permease protein MsbA OS=Stenotrophomonas maltophilia (strain K279a) GN=msbA PE=3 SV=1 - [B2FK21_STRMK]**	**0.01**	**1.88**
B2FK77	Putative transmembrane CorC/HlyC family transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1704 PE=4 SV=1 - [B2FK77_STRMK]	0.00	1.50
B2FK79	Putative LOW-AFFINITY INORGANIC PHOSPHATE TRANSPORTER INTEGRAL MEMBRANE PROTEIN PITA OS=Stenotrophomonas maltophilia (strain K279a) GN=pitA PE=4 SV=1 - [B2FK79_STRMK]	0.00	1.81
B2FK88	Enolase OS=Stenotrophomonas maltophilia (strain K279a) GN=eno PE=3 SV=1 - [ENO_STRMK]	<0.05	>100
B2FK95	Protein-L-isoaspartate O-methyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=pcm PE=3 SV=1 - [PIMT_STRMK]	0.02	1.59
B2FKA2	ATP-dependent zinc metalloprotease FtsH OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsH PE=3 SV=1 - [B2FKA2_STRMK]	0.04	1.25
B2FKJ7	50S ribosomal protein L9 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplI PE=3 SV=1 - [RL9_STRMK]	0.01	0.45
B2FKK2	Asparagine--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=asnS PE=3 SV=1 - [B2FKK2_STRMK]	<0.05	>100
B2FKL0	Putative haloacid dehalogenase hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4308 PE=4 SV=1 - [B2FKL0_STRMK]	0.02	0.62
B2FKS9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4386 PE=4 SV=1 - [B2FKS9_STRMK]	<0.05	>100
B2FKV7	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0483 PE=4 SV=1 - [B2FKV7_STRMK]	0.01	0.59
B2FKW2	Pyruvate dehydrogenase E1 component OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0490 PE=4 SV=1 - [B2FKW2_STRMK]	0.00	1.96
B2FL08	Putative transmembrane anchor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0538 PE=4 SV=1 - [B2FL08_STRMK]	0.00	0.15
B2FL09	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0539 PE=4 SV=1 - [B2FL09_STRMK]	0.00	0.34
B2FL10	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0540 PE=4 SV=1 - [B2FL10_STRMK]	0.01	0.69
B2FL20	Putative ZINC METALLOpeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0550 PE=4 SV=1 - [B2FL20_STRMK]	0.04	1.59
B2FL73	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1785 PE=4 SV=1 - [B2FL73_STRMK]	0.00	1.99
B2FL82	Putative ABC transporter ATP-binding subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1794 PE=4 SV=1 - [B2FL82_STRMK]	>0.05	0.00
B2FL84	Putative succinate dehydrogenase cytochrome b-556 subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=sdhC PE=4 SV=1 - [B2FL84_STRMK]	0.01	1.70
B2FLA1	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3164 PE=4 SV=1 - [B2FLA1_STRMK]	0.04	1.68
B2FLA7	Putative multidrug resistance efflux pump OS=Stenotrophomonas maltophilia (strain K279a) GN=smeH PE=4 SV=1 - [B2FLA7_STRMK]	0.03	1.28
B2FLD1	Putative 2-oxoglutarate dehydrogenase E1 component OS=Stenotrophomonas maltophilia (strain K279a) GN=sucA PE=4 SV=1 - [B2FLD1_STRMK]	0.04	1.95
B2FLD2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex OS=Stenotrophomonas maltophilia (strain K279a) GN=sucB PE=3 SV=1 - [B2FLD2_STRMK]	0.00	1.80
B2FLE0	Asparagine synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3206 PE=4 SV=1 - [B2FLE0_STRMK]	0.02	1.67
B2FLE4	Putative outer membrane antigen protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3210 PE=4 SV=1 -	0.01	0.44

	[B2FLE4_STRMK]		
B2FLE9	Putative outer membrane antigen lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3215 PE=4 SV=1 - [B2FLE9_STRMK]	0.03	1.60
B2FLG2	Putative peptidyl dipeptidase/oligopeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3229 PE=3 SV=1 - [B2FLG2_STRMK]	0.00	2.07
B2FLG5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3232 PE=4 SV=1 - [B2FLG5_STRMK]	0.04	0.83
B2FLH4	Putative transmembrane repetitive protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3241 PE=4 SV=1 - [B2FLH4_STRMK]	0.00	2.32
B2FLQ1	D-amino acid dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=dadA PE=3 SV=1 - [DADA_STRMK]	0.05	0.60
B2FLR8	Putative vitamin B12 receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0585 PE=3 SV=1 - [B2FLR8_STRMK]	0.01	0.76
B2FLT5	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0602 PE=3 SV=1 - [B2FLT5_STRMK]	0.02	0.46
B2FLU9	Putative ABC transporter component, polysaccharide related OS=Stenotrophomonas maltophilia (strain K279a) GN=wzt PE=4 SV=1 - [B2FLU9_STRMK]	0.02	1.42
B2FLV2	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=wxocA PE=4 SV=1 - [B2FLV2_STRMK]	0.04	1.23
B2FLV3	Putative glycosyltransferase, fusion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=wxocBC PE=4 SV=1 - [B2FLV3_STRMK]	0.05	1.78
B2FLV9	Putative FMN amine oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0629 PE=4 SV=1 - [B2FLV9_STRMK]	0.05	1.43
B2FLW2	Chaperone protein HtpG OS=Stenotrophomonas maltophilia (strain K279a) GN=htpG PE=3 SV=1 - [B2FLW2_STRMK]	0.05	1.66
B2FLX9	Putative outer membrane lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1826 PE=3 SV=1 - [B2FLX9_STRMK]	0.02	1.47
B2FM92	Ribonuclease E OS=Stenotrophomonas maltophilia (strain K279a) GN=rnE PE=3 SV=1 - [B2FM92_STRMK]	0.03	1.72
B2FMF8	Putative GAF/GGDEF/EAL domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3318 PE=4 SV=1 - [B2FMF8_STRMK]	0.05	0.65
B2FMI1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4498 PE=4 SV=1 - [B2FMI1_STRMK]	0.01	1.49
B2FMI9	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4506 PE=4 SV=1 - [B2FMI9_STRMK]	0.01	0.55
B2FMJ3	Putative ABC transmembrane permease component transport system protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4510 PE=4 SV=1 - [B2FMJ3_STRMK]	0.05	0.71
B2FMJ4	Putative ATP-binding transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4511 PE=4 SV=1 - [B2FMJ4_STRMK]	>0.05	0.00
B2FMQ7	Putative glycosyltransferase protein OS=Stenotrophomonas maltophilia (strain K279a) GN=wxocA PE=4 SV=1 - [B2FMQ7_STRMK]	0.01	1.55
B2FMR0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0660 PE=4 SV=1 - [B2FMR0_STRMK]	0.00	0.76
B2FMR2	Putative permease component of ABC transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0662 PE=4 SV=1 - [B2FMR2_STRMK]	0.01	0.69
B2FMR8	Putative transmembrane CDP-diacylglycerol--serine O-phosphatidyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0669 PE=3 SV=1 - [B2FMR8_STRMK]	0.00	1.88
B2FMT0	Putative thiol:disulfide interchange protein DsbC OS=Stenotrophomonas maltophilia (strain K279a) GN=dsbC PE=4 SV=1 - [B2FMT0_STRMK]	0.03	1.55

B2FN02	Protein translocase subunit SecD OS=Stenotrophomonas maltophilia (strain K279a) GN=secD PE=3 SV=1 - [B2FN02_STRMK]	0.00	1.72
B2FN03	Protein-export membrane protein SecF OS=Stenotrophomonas maltophilia (strain K279a) GN=secF PE=3 SV=1 - [B2FN03_STRMK]	0.01	1.38
B2FN23	Putative transmembrane TraB family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2036 PE=4 SV=1 - [B2FN23_STRMK]	0.03	1.43
B2FN37	Putative N-acetylmuramoyl-L-alanine amidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3330 PE=4 SV=1 - [B2FN37_STRMK]	0.02	0.70
B2FN47	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3340 PE=3 SV=1 - [B2FN47_STRMK]	0.00	0.51
B2FN59	Conserved hypothetical repetitive protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3358 PE=4 SV=1 - [B2FN59_STRMK]	0.00	1.79
B2FN82	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3381 PE=4 SV=1 - [B2FN82_STRMK]	<0.05	>100
B2FN86	Polyribonucleotide nucleotidyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=pnp PE=3 SV=1 - [PNP_STRMK]	0.04	1.44
B2FN87	30S ribosomal protein S15 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsO PE=3 SV=1 - [RS15_STRMK]	0.02	1.54
B2FN93	NADH-quinone oxidoreductase subunit N OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoN PE=3 SV=1 - [B2FN93_STRMK]	0.04	1.47
B2FN94	Putative NADH dehydrogenase I chain M OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoM PE=4 SV=1 - [B2FN94_STRMK]	0.00	1.86
B2FN95	Putative NADH-ubiquinone oxidoreductase I chain L OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoL PE=4 SV=1 - [B2FN95_STRMK]	0.00	2.20
B2FN97	Putative transmembrane ankyrin repeat protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4553 PE=4 SV=1 - [B2FN97_STRMK]	0.00	1.59
B2FN99	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4555 PE=4 SV=1 - [B2FN99_STRMK]	>0.05	0.00
B2FNC5	Putative dipeptidyl peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4581 PE=4 SV=1 - [B2FNC5_STRMK]	0.03	1.33
B2FND5	Universal stress protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4591 PE=3 SV=1 - [B2FND5_STRMK]	0.02	0.48
B2FND7	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4593 PE=4 SV=1 - [B2FND7_STRMK]	0.00	0.28
B2FNE2	50S ribosomal protein L33 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpmG PE=3 SV=1 - [RL33_STRMK]	>0.05	0.00
B2FNE3	50S ribosomal protein L28 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpmB PE=3 SV=1 - [RL28_STRMK]	0.03	1.67
B2FNF1	Putative transmembrane efflux pump protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smmQ PE=4 SV=1 - [B2FNF1_STRMK]	0.00	1.73
B2FNF2	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4609 PE=4 SV=1 - [B2FNF2_STRMK]	<0.05	>100
B2FNF4	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4611 PE=4 SV=1 - [B2FNF4_STRMK]	0.05	1.34
B2FNI0	Putative general secretion pathway protein L OS=Stenotrophomonas maltophilia (strain K279a) GN=pefL PE=4 SV=1 - [B2FNI0_STRMK]	0.00	0.54
B2FNJ1	Putative pili chaperone protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0707 PE=3 SV=1 - [B2FNJ1_STRMK]	0.03	2.55
B2FNJ2	Putative outer membrane usher protein mrkc OS=Stenotrophomonas maltophilia (strain K279a) GN=mrkC PE=4 SV=1 - [B2FNJ2_STRMK]	0.00	2.54

B2FNJ3	Putative fimbria adhesin protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0709 PE=4 SV=1 - [B2FNJ3_STRMK]	0.00	3.45
B2FNK0	Putative ABC transporter component protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0716 PE=3 SV=1 - [B2FNK0_STRMK]	0.00	0.66
B2FNL0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0726 PE=4 SV=1 - [B2FNL0_STRMK]	0.00	0.28
B2FNM5	Putative LppC family lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0742 PE=4 SV=1 - [B2FNM5_STRMK]	0.00	1.29
B2FNP0	Cell division protein FtsQ OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsQ PE=3 SV=1 - [B2FNP0_STRMK]	0.04	0.58
B2FNQ0	Putative epimerase/dehydratase polysaccharide-related biosynthesis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=wbil PE=4 SV=1 - [B2FNQ0_STRMK]	0.00	1.69
B2FNQ5	Nucleoside diphosphate kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=ndk PE=3 SV=1 - [NDK_STRMK]	0.00	2.72
B2FNR0	Outer membrane protein assembly factor BamB OS=Stenotrophomonas maltophilia (strain K279a) GN=bamB PE=3 SV=1 - [B2FNR0_STRMK]	0.01	1.31
B2FNS0	Inosine-5'-monophosphate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=guaB PE=3 SV=1 - [B2FNS0_STRMK]	0.05	1.62
B2FNX6	NADH-quinone oxidoreductase subunit D OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoD PE=3 SV=1 - [NUOD_STRMK]	0.01	1.48
B2FNX7	NADH-quinone oxidoreductase subunit C OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoC PE=3 SV=1 - [NUOC_STRMK]	0.03	1.29
B2FNY8	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=accD PE=3 SV=2 - [ACCD_STRMK]	0.02	1.65
B2FP19	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3449 PE=4 SV=1 - [B2FP19_STRMK]	0.00	0.29
B2FP42	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3472 PE=4 SV=1 - [B2FP42_STRMK]	0.00	3.94
B2FP45	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4632 PE=4 SV=1 - [B2FP45_STRMK]	0.00	2.48
B2FP84	Putative transmembrane mce related protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4672 PE=4 SV=1 - [B2FP84_STRMK]	0.05	1.66
B2FP90	Putative RmuC family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4678 PE=4 SV=1 - [B2FP90_STRMK]	0.02	1.37
B2FP97	Putative amino-acid transporter transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4685 PE=4 SV=1 - [B2FP97_STRMK]	0.03	1.62
B2FPA8	Cell division protein FtsZ OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsZ PE=3 SV=1 - [B2FPA8_STRMK]	0.02	0.71
B2FPD1	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0784 PE=4 SV=1 - [B2FPD1_STRMK]	>0.05	0.00
B2FPE2	Putative exported heme receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=huvA PE=3 SV=1 - [B2FPE2_STRMK]	0.00	1.70
B2FPN5	Putative TonB-dependent outer membrane receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2179 PE=3 SV=1 - [B2FPN5_STRMK]	0.05	0.59
B2FPN6	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2180 PE=4 SV=1 - [B2FPN6_STRMK]	<0.05	>100
B2FPS6	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3494 PE=4 SV=1 - [B2FPS6_STRMK]	0.00	0.51
B2FPV5	Putative autotransporter subtilisin-like protease OS=Stenotrophomonas maltophilia (strain K279a) GN=sphB PE=4 SV=1 - [B2FPV5_STRMK]	0.01	0.63

B2FPY0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3550 PE=4 SV=1 - [B2FPY0_STRMK]	0.00	0.15
B2FPY1	Putative signal peptidase I OS=Stenotrophomonas maltophilia (strain K279a) GN=lepB PE=3 SV=1 - [B2FPY1_STRMK]	0.00	1.53
B2FPY7	Putative alkyl hydroperoxide reductase subunit c OS=Stenotrophomonas maltophilia (strain K279a) GN=ahpC PE=4 SV=1 - [B2FPY7_STRMK]	0.01	2.20
B2FQ10	UPF0761 membrane protein Smlt0865 OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0865 PE=3 SV=1 - [Y865_STRMK]	0.00	0.57
B2FQ28	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0885 PE=3 SV=1 - [B2FQ28_STRMK]	0.01	0.63
B2FQ35	50S ribosomal protein L1 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplA PE=3 SV=1 - [RL1_STRMK]	0.05	0.58
B2FQ39	DNA-directed RNA polymerase subunit beta' OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoC PE=3 SV=1 - [RPOC_STRMK]	0.00	3.33
B2FQ42	Elongation factor G OS=Stenotrophomonas maltophilia (strain K279a) GN=fusA PE=3 SV=1 - [EFG_STRMK]	0.03	2.14
B2FQ45	50S ribosomal protein L3 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplC PE=3 SV=1 - [RL3_STRMK]	0.01	0.61
B2FQ46	50S ribosomal protein L4 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplD PE=3 SV=1 - [RL4_STRMK]	0.02	0.79
B2FQ54	**Putative secretion protein-HlyD family OS=Stenotrophomonas maltophilia (strain K279a) GN=smeY PE=4 SV=1 - [B2FQ54_STRMK]**	**>0.05**	**0.00**
B2FQ55	**Putative multidrug ACR family efflux system OS=Stenotrophomonas maltophilia (strain K279a) GN=smeZ PE=4 SV=1 - [B2FQ55_STRMK]**	**>0.05**	**0.21**
B2FQ57	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2204 PE=4 SV=1 - [B2FQ57_STRMK]	0.04	1.37
B2FQ64	Putative ferrous iron transport protein B OS=Stenotrophomonas maltophilia (strain K279a) GN=feoB PE=4 SV=1 - [B2FQ64_STRMK]	0.02	1.64
B2FQC5	Putative subfamily S1C unassigned peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3553 PE=4 SV=1 - [B2FQC5_STRMK]	0.00	2.91
B2FQD7	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3567 PE=4 SV=1 - [B2FQD7_STRMK]	0.04	2.39
B2FQD9	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3569 PE=4 SV=1 - [B2FQD9_STRMK]	0.00	2.15
B2FQG3	Protein HflC OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3595 PE=3 SV=1 - [B2FQG3_STRMK]	0.02	1.36
B2FQG4	Putative HflK protein OS=Stenotrophomonas maltophilia (strain K279a) GN=hflK PE=4 SV=1 - [B2FQG4_STRMK]	0.00	1.90
B2FQJ5	50S ribosomal protein L24 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplX PE=3 SV=1 - [RL24_STRMK]	0.01	0.35
B2FQK4	Protein translocase subunit SecY OS=Stenotrophomonas maltophilia (strain K279a) GN=secY PE=3 SV=1 - [B2FQK4_STRMK]	0.01	1.61
B2FQK8	DNA-directed RNA polymerase subunit alpha OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoA PE=3 SV=1 - [RPOA_STRMK]	0.03	1.43
B2FQL6	Putative GTP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=typA PE=4 SV=1 - [B2FQL6_STRMK]	0.00	0.54
B2FQL8	Malate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=mdh PE=3 SV=1 - [MDH_STRMK]	0.02	1.90
B2FQM8	Putative outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0955 PE=3 SV=1 - [B2FQM8_STRMK]	0.00	1.51
B2FQN3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0960 PE=4 SV=1 - [B2FQN3_STRMK]	0.00	0.15

B2FQN5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0962 PE=4 SV=1 - [B2FQN5_STRMK]	0.02	2.28
B2FQQ5	Putative isocitrate/isopropylmalate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0982 PE=3 SV=1 - [B2FQQ5_STRMK]	0.01	2.30
B2FQR3	ATP-dependent Clp protease ATP-binding subunit ClpX OS=Stenotrophomonas maltophilia (strain K279a) GN=clpX PE=3 SV=1 - [CLPX_STRMK]	0.00	2.30
B2FR07	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3644 PE=4 SV=1 - [B2FR07_STRMK]	0.00	2.02
B2FR08	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3645 PE=3 SV=1 - [B2FR08_STRMK]	0.00	0.20
B2FR11	Putative acyl-coenzyme A dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=fadE PE=4 SV=1 - [B2FR11_STRMK]	0.02	1.56
B2FR23	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3660 PE=4 SV=1 - [B2FR23_STRMK]	0.02	0.69
B2FR43	Putative penicillin-binding protein 1B OS=Stenotrophomonas maltophilia (strain K279a) GN=mrcB PE=4 SV=1 - [B2FR43_STRMK]	0.00	2.01
B2FR61	Putative phosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0999 PE=4 SV=1 - [B2FR61_STRMK]	0.03	1.37
B2FR62	Putative autotransporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1001 PE=4 SV=1 - [B2FR62_STRMK]	0.01	0.65
B2FR78	Putative outer membrane lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1018 PE=4 SV=1 - [B2FR78_STRMK]	0.00	1.78
B2FRM9	Protein TolB OS=Stenotrophomonas maltophilia (strain K279a) GN=tolB PE=3 SV=1 - [B2FRM9_STRMK]	0.00	1.74
B2FRP8	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3725 PE=3 SV=1 - [B2FRP8_STRMK]	0.00	0.59
B2FRP9	Putative transmembrane transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3726 PE=4 SV=1 - [B2FRP9_STRMK]	0.05	0.87
B2FRQ4	Putative heat shock chaperone ClpB OS=Stenotrophomonas maltophilia (strain K279a) GN=clpB PE=3 SV=1 - [B2FRQ4_STRMK]	0.01	2.30
B2FRQ5	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3733 PE=4 SV=1 - [B2FRQ5_STRMK]	<0.05	>100
B2FRR0	Putative lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3739 PE=4 SV=1 - [B2FRR0_STRMK]	0.01	0.43
B2FRR1	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3740 PE=3 SV=1 - [B2FRR1_STRMK]	0.00	0.48
B2FRS9	Putative pilus-assembly protein OS=Stenotrophomonas maltophilia (strain K279a) GN=pilG PE=3 SV=1 - [B2FRS9_STRMK]	>0.05	0.00
B2FRW7	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1115 PE=4 SV=1 - [B2FRW7_STRMK]	0.01	1.66
B2FS15	Putative histone-like protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1164 PE=4 SV=1 - [B2FS15_STRMK]	0.01	1.89
B2FSD1	tRNA (guanine-N(7)-)-methyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=trmB PE=3 SV=1 - [TRMB_STRMK]	>0.05	>100
B2FSE2	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeN PE=4 SV=1 - [B2FSE2_STRMK]	0.03	1.97
B2FSE3	Putative HlyD-family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeM PE=4 SV=1 - [B2FSE3_STRMK]	0.01	1.50
B2FSG4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3812 PE=4 SV=1 - [B2FSG4_STRMK]	0.00	2.16
B2FSG7	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3815 PE=4 SV=1 - [B2FSG7_STRMK]	0.01	1.63
B2FSH0	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3818 PE=4 SV=1 - [B2FSH0_STRMK]	0.05	1.64

B2FSH1	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3819 PE=4 SV=1 - [B2FSH1_STRMK]	0.00	1.76
B2FSH7	Putative PilM protein (Type 4 fimbrial biogenesis protein) OS=Stenotrophomonas maltophilia (strain K279a) GN=pilM PE=4 SV=1 - [B2FSH7_STRMK]	0.02	0.26
B2FSI7	Citrate synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=gltA PE=3 SV=1 - [B2FSI7_STRMK]	0.02	2.54
B2FSQ7	Putative TonB-dependent receptor for Fe(III)-coprogen, Fe(III)-ferrioxamine B and Fe(III)-rhodotrulic acid OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1233 PE=3 SV=1 - [B2FSQ7_STRMK]	0.02	1.67
B2FSQ8	Probable malate:quinone oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=mqo PE=3 SV=1 - [MQO_STRMK]	0.03	0.57
B2FT44	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3882 PE=4 SV=1 - [B2FT44_STRMK]	0.03	1.61
B2FT45	Putative transmembrane DedA family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3883 PE=4 SV=1 - [B2FT45_STRMK]	0.02	1.40
B2FT59	Putative extracellular heme-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3898 PE=3 SV=1 - [B2FT59_STRMK]	0.02	1.67
B2FT66	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3905 PE=3 SV=1 - [B2FT66_STRMK]	0.01	0.53
B2FT88	Putative biopolymer transport exbB protein OS=Stenotrophomonas maltophilia (strain K279a) GN=exbB1 PE=3 SV=1 - [B2FT88_STRMK]	0.01	1.28
B2FTA3	Putative exported peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1246 PE=4 SV=1 - [B2FTA3_STRMK]	0.00	2.45
B2FTA6	Cell division topological specificity factor OS=Stenotrophomonas maltophilia (strain K279a) GN=minE PE=3 SV=1 - [MINE_STRMK]	0.00	2.33
B2FTB8	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1263 PE=4 SV=1 - [B2FTB8_STRMK]	0.04	0.61
B2FTC4	Putative transmembrane protein, similar to that required for pathogenicity of Xanthomonas campestris in plants OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1270 PE=4 SV=1 - [B2FTC4_STRMK]	0.02	1.46
B2FTD2	50S ribosomal protein L27 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpmA PE=3 SV=1 - [RL27_STRMK]	0.02	0.79
B2FTI8	Putative lipid II flippase MurJ OS=Stenotrophomonas maltophilia (strain K279a) GN=mviN PE=3 SV=1 - [B2FTI8_STRMK]	0.01	1.55
B2FTN0	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2678 PE=4 SV=1 - [B2FTN0_STRMK]	0.00	1.79
B2FTS1	Putative drug-resistance cell envelope-related protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeP PE=4 SV=1 - [B2FTS1_STRMK]	0.02	2.06
B2FTS4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=pcm PE=4 SV=1 - [B2FTS4_STRMK]	0.04	0.60
B2FTS5	Protein CyaE OS=Stenotrophomonas maltophilia (strain K279a) GN=tolC PE=3 SV=1 - [B2FTS5_STRMK]	0.04	0.80
B2FTS7	Putative 3-deoxy-D-manno-octulosonic-acid transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=kdtA PE=4 SV=1 - [B2FTS7_STRMK]	0.01	1.69
B2FTU3	Putative phosphate selective porin OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3950 PE=4 SV=1 - [B2FTU3_STRMK]	0.04	1.25
B2FTU5	Putative acetoacetyl-CoA reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=phbB PE=3 SV=1 - [B2FTU5_STRMK]	0.00	3.72
B2FTY7	Thiol:disulfide interchange protein OS=Stenotrophomonas maltophilia (strain K279a) GN=dsbA PE=3 SV=1 - [B2FTY7_STRMK]	0.04	1.72
B2FTY8	Putative thiol:disulfide interchange protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3994 PE=4 SV=1 - [B2FTY8_STRMK]	0.01	1.60
B2FU42	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0083 PE=3 SV=1 - [B2FU42_STRMK]	0.03	0.73

B2FU60	Glycerol-3-phosphate acyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=plsB PE=3 SV=1 - [B2FU60_STRMK]	0.03	1.67
B2FU91	Putative outer membrane autotransporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1350 PE=4 SV=1 - [B2FU91_STRMK]	>0.05	0.00
B2FUA0	Putative transmembrane ubiquinol oxidase subunit 2 OS=Stenotrophomonas maltophilia (strain K279a) GN=qoxA PE=4 SV=1 - [B2FUA0_STRMK]	0.01	1.73
B2FUB3	30S ribosomal protein S16 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsP PE=3 SV=1 - [RS16_STRMK]	0.00	1.90
B2FUE0	Putative outer membrane efflux protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1407 PE=4 SV=1 - [B2FUE0_STRMK]	0.00	0.70
B2FUL2	Putative respiratory nitrate reductase alpha chain OS=Stenotrophomonas maltophilia (strain K279a) GN=narG PE=4 SV=1 - [B2FUL2_STRMK]	0.01	2.75
B2FUN9	Putative TonB-dependent receptor for Fe(III)-coprogen, Fe(III)-ferrioxamine B and Fe(III)-rhodotrulic acid OS=Stenotrophomonas maltophilia (strain K279a) GN=fhuE PE=3 SV=1 - [B2FUN9_STRMK]	0.01	1.37
B2FUP4	Putative transglycosylase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4007 PE=4 SV=1 - [B2FUP4_STRMK]	0.01	1.94
B2FUP9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4012 PE=4 SV=1 - [B2FUP9_STRMK]	>0.05	0.00
B2FUR1	Putative TonB-dependent outer membrane receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4026 PE=3 SV=1 - [B2FUR1_STRMK]	0.00	0.43
B2FUS7	Putative exported tail-specific protease OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4045 PE=3 SV=1 - [B2FUS7_STRMK]	0.00	2.13
B2FUT1	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4049 PE=4 SV=1 - [B2FUT1_STRMK]	0.04	0.75
B2FUT2	Putative penicillin-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=dacC PE=3 SV=1 - [B2FUT2_STRMK]	0.01	1.55
B2FUT3	Putative exported rare lipoprotein A OS=Stenotrophomonas maltophilia (strain K279a) GN=rlpA PE=3 SV=1 - [B2FUT3_STRMK]	0.00	0.70
B2FUT4	Putative murein hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=mltB PE=4 SV=1 - [B2FUT4_STRMK]	0.00	1.42
B2FUT7	Putative penicillin-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=mrdA PE=4 SV=1 - [B2FUT7_STRMK]	0.01	1.67
B2FUT9	Putative rod shape-determining protein OS=Stenotrophomonas maltophilia (strain K279a) GN=mreC PE=4 SV=1 - [B2FUT9_STRMK]	0.03	1.37
B2FUU0	Putative rod shape-determining protein OS=Stenotrophomonas maltophilia (strain K279a) GN=mreB PE=4 SV=1 - [B2FUU0_STRMK]	0.04	0.65
B2FUU5	Putative PEPTIDASE OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4064 PE=4 SV=1 - [B2FUU5_STRMK]	0.01	1.51
B2FUV1	**Putative multidrug resistance outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeF PE=4 SV=1 - [B2FUV1_STRMK]**	**0.00**	**4.00**
B2FUV2	**Putative acriflavin resistance protein B OS=Stenotrophomonas maltophilia (strain K279a) GN=smeE PE=4 SV=1 - [B2FUV2_STRMK]**	**0.00**	**4.74**
B2FUV3	**Putative acriflavin resistance protein A OS=Stenotrophomonas maltophilia (strain K279a) GN=smeD PE=4 SV=1 - [B2FUV3_STRMK]**	**0.00**	**2.42**
B2FUV6	ATP-dependent protease ATPase subunit HslU OS=Stenotrophomonas maltophilia (strain K279a) GN=hslU PE=3 SV=1 - [B2FUV6_STRMK]	0.00	3.46

5 Characterisation of β-lactamase inhibitors in *S. maltophilia*

5.1 Introduction

β-lactamases are the most commonly encountered cause of resistance to β-lactams, which are the most frequently prescribed class of antibacterial drug world-wide (285-287). β-lactamases render β-lactams inactive through catalysing efficient hydrolysis of the β-lactam ring (288, 289). There are many hundreds of known β-lactamases, which are grouped based on sequence and mechanism into the serine β-lactamase (SBL) classes A, C and D, and the metallo-β-lactamase (MBL) subclasses B1, B2 and B3) (290, 291). Broad-spectrum, clinically useful β-lactamase inhibitors are being sought, but the varying chemistries and active site architectures of the different β-lactamase classes makes the development of cross-class inhibitors extremely challenging (114, 292, 293).

Clavulanic acid (**Figure 5.1, *top***) is a well-established clinically deployed β-lactam-based inhibitor of, principally, class A SBLs. Clavulanic acid is used in combination with penicillin derivatives such as amoxicillin (**Figure 1.4**) and ticarcillin (**Figure 1.4**), whose bactericidal effects improve against some β-lactamase-carrying isolates of species such as *E. coli* and *K. pneumoniae* (294-297). Clavulanic acid is an irreversible inhibitor of class A enzymes, whose activity arises from fragmentation of the acyl-enzyme complex formed by reaction with the active-site serine nucleophile, to generate a near permanently inactivated species (136, 298, 299). In contrast, avibactam (formerly NXL104), a recently introduced relatively broad spectrum non-β-lactam-based SBL inhibitor contains a diazobicyclo heterocyclic core structure which reversibly acylates SBLs. The potency of avibactam against class A, C and some class D SBLs is attributed to the stabilization of the carbamoyl complex due to interactions with polar residues present in the active sites, with de-acylation preferentially occurring due to recyclization rather than hydrolytic turnover (300). This recyclization results in release of intact active inhibitor rather than an inactive hydrolysis product (**Figure 5.1**, ***middle***) (272, 301-304). Avibactam has recently been licenced for clinical use in partnership with the oxy-amino cephalosporin ceftazidime, though the combination is not universally efficacious and has no useful activity against MBL-producing bacteria (302, 305).

Boronic acid-based compounds have long been studied as potential SBL inhibitors (306-308) but, in most cases, are ineffective against MBL targets. For example, the monocyclic boronate vaborbactam (RPX7009) (**Figure 5.1, *bottom left***), is effective against Class A, C and D β-lactamases, but not MBLs (309). However, we recently demonstrated that bicyclic boronate scaffolds can act as potent inhibitors of multiple SBL classes as well as subclass B1 MBLs (293). Accordingly, one method of overcoming the poor activity of ceftazidime/avibactam (**Figure 1.6**) against MBL producing bacteria would be to combine ceftazidime with a bicyclic boronate inhibitor, such as **2** (**Figure 5.1, *bottom right***), or **1**, which is derived from the same scaffold (310). Together, **1** and **2** represent the closest approach to a pan-β-lactamase inhibitor that has, to-date, been reported in the literature (310). Another bicyclic boronate β-lactamase inhibitor (VNRX-5133) has recently completed phase 1 clinical trials (311).

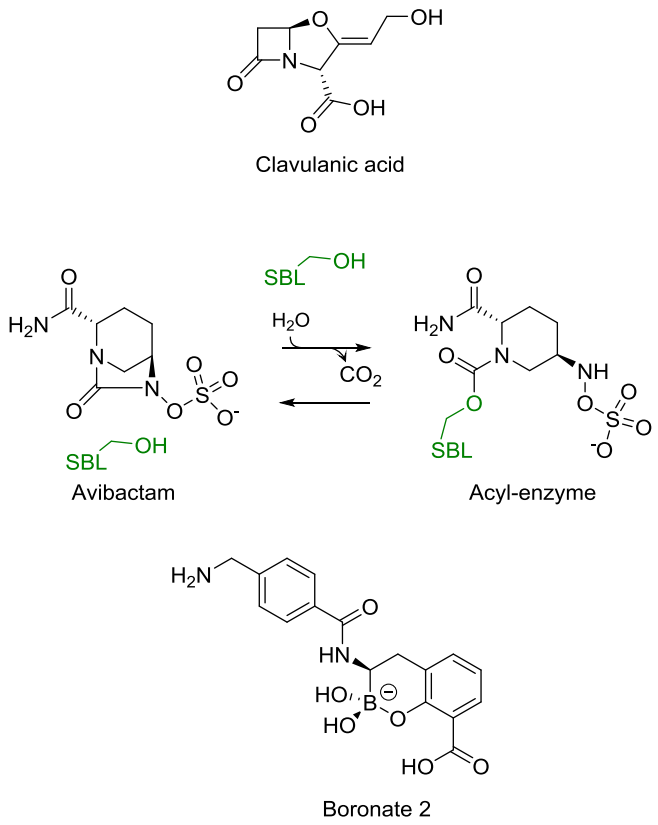


Figure 5.1 Chemical structures of β-lactamase inhibitors

Top, clavulanic acid. Middle, avibactam and the acyl-enzyme complex formed on reaction of avibactam with SBLs. Bottom, cyclic boronate **2**

Thiol-based compounds provided the first insights in MBL inhibition (111, 312-314). Since their discovery, part of the MBL inhibitor design has been focused on

compounds that include metal-chelating moieties (315) that displaces zinc atom from the active site to impede β-lactam hydrolysis (316). Rhodanine-based compounds possess a thiazolidine core with two-chelating moieties, sulphur and a nitrogen atom. In fact, rhodanines and their derivatives have shown to inhibit MBLs (IMP-1, VIM-2, NDM-1) with submicromolar *K*i values (316, 317). Other zinc-binding inhibitors include a phosphonate moiety that have shown the potential to become broad-spectrum MBLs inhibitors (318). Mercaptophosphonate compounds have shown to inhibit VIM-4, CphA, and L1 with *K*i values ranging from 0.4 to > 400 μM (319) (**Figure 1.21**).

While *S. maltophilia* possesses multiple efflux systems (283, 320-322) that reduce the net rate of entry for many antimicrobials, as discussed in **Chapter 4**, β-lactam resistance arises primarily from production of two β-lactamases, a subclass B3 MBL "L1", which hydrolyses all β-lactams except for the monobactam, aztreonam (**Figure 1.8**), and the class A Extended Spectrum SBL (ESBL) "L2", which hydrolyses all first to third generation cephalosporins, all penicillins, and aztreonam (153, 156, 157). The combination of L1 and L2, therefore renders *S. maltophilia* resistant to all β-lactam antibiotics although in clinical practice, ceftazidime can be useful, because most clinical isolates do not produce enough β-lactamase to give resistance (323, 324). However, ceftazidime resistant mutants rapidly emerge as discussed in **Chapter 3**. Accordingly, *S. maltophilia* represents one of the most challenging targets for β-lactam/β-lactamase inhibitor combinations.

Here, we aimed to provide kinetic and structural insights of the interaction of *S. maltophilia* β-lactamases and β-lactamase inhibitors as well as testing various β-lactam/β-lactamase inhibitor combinations against extensively drug resistant clinical *S. maltophilia* isolates and characterisation of acquired resistance to these combinations.

5.2 Results & discussion

5.2.1 β-lactamase inhibitors restore aztreonam, but not meropenem activity against *S. maltophilia*

As a prelude to investigating the effects of β-lactamase inhibitors, we first evaluated the hydrolysis of a range of candidate β-lactams *in vitro* by purified L1 (subclass B3 MBL) and L2 (class A ESBL) under steady state conditions. These data (**Table 5.1**) reveal the carbapenem meropenem (**Figure 1.7**) to be predominantly a

substrate for L1, with L2 showing only weak hydrolytic activity, the monobactam aztreonam to be a substrate for L2 only, and that both L1 and L2 can hydrolyse the oxyamino-cephalosporin ceftazidime with similar efficiencies.

Table 5.1 Kinetic data for β-lactams tested against metallo L1 and serine L2 *S. maltophilia* β-lactamases.

Enzyme	Substrate	[E] (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}.s^{-1}$) x10^{-3}
L1	Ceftazidime	0.5	259.5	1.67	6.4
	Aztreonam	0.5	-	-	-
	Meropenem	10	105	23.8	227
	FC5	0.05	29.6	146	4,932
L2	Ceftazidime	0.5	548.5	1.88	3.4
	Aztreonam	0.5	119.4	0.08	0.67
	Meropenem	0.625	28.83	0.028	0.97
	FC5	0.05	17.9	208.6	11,653

We next tested the ability of three β-lactamase inhibitors: clavulanic acid, avibactam and the bicyclic boronate 2 (each at 2 mg/L) to potentiate the activity of the target β-lactams against *S. maltophilia* (**Table 5.2**) All three inhibitors reversed aztreonam, but not meropenem resistance in ceftazidime susceptible clinical isolates (K279a, CI-20, CI-29). Furthermore, all three inhibitors reversed ceftazidime and aztreonam, but not meropenem, resistance in a ceftazidime-resistant L1/L2 hyper-producing mutant (K CAZ 10), derived from K279a (**Table 5.2**) (214). Interestingly, all three inhibitors were unable to restore ceftazidime susceptibility in a ceftazidime resistant L1/L2 hyper-producing clinical isolate (CI-31), but they all restored aztreonam susceptibility in CI-31 (**Table 5.2**).

Table 5.2 Minimum Inhibitory Concentrations (mg/L) of β-lactams against *S. maltophilia* in the presence of β-lactamase inhibitors used at 2 mg/L

	Ceftazidime				Aztreonam				Meropenem			
	-	+CLA	+BOR	+AVI	-	+CLA	+BOR	+AVI	-	+CLA	+BOR	+AVI
K279a	4	4	0.5	1	128	1	1	1	8	32	4	16
CI-20	16	16	2	4	128	4	2	2	64	32	8	64
CI-29	8	4	0.5	1	128	1	1	1	32	16	8	32
K CAZ 10	64	8	4	8	256	0.5	1	1	64	8	16	64
KCAZ14	64	32	8	2	256	-	2	1	128	32	32	128
CI-31	256	128	128	128	256	2	4	4	256	256	256	256
K AMI 32	2	1	1	0.5	128	0.5	1	0.5	4	8	4	16
K MOX 8	4	1	0.5	0.5	128	0.25	1	0.5	4	8	8	16

Shaded values indicate clinically relevant resistance according to CLSI breakpoints (325)

(-) Inconsistent values

CLA, clavulanic acid; BOR, bicyclic boronate **2**; AVI, avibactam

Efflux pumps play a major role in antimicrobial resistance in *S. maltophilia* (130, 173). Thus, to investigate the possible effect of multi-drug efflux pumps on β-lactamase inhibitor efficacy, we tested the effect on K AMI 32 and K MOX 8, SmeYZ and SmeDEF hyperproducers, respectively as described in **Chapter 4**. All three β-lactamase inhibitors retained full activity against these efflux pump hyper-producing mutants (**Table 5.2**) ruling out efflux (or at least SmeDEF and SmeYZ) as a contributing factor to the observed variation in efficacy of the various β-lactam/β-lactamase inhibitor combinations.

5.2.2 The bicyclic boronate 2 does not inhibit the *S. maltophilia* L1 MBL

Based on these *in vitro* data we conclude that the bicyclic boronate **2** acts against *S. maltophilia* in a similar fashion to avibactam and clavulanic acid: it reverses aztreonam and, when due to L1/L2 hyperproduction, ceftazidime resistance (**Table 5.2**). As **2** has been shown to inhibit multiple MBLs (293), we anticipated that it might also inhibit L1, but the fact that **2** does not reverse resistance to meropenem (**Table**

5.2), which is predominantly hydrolysed by L1 (**Table 5.1**) suggests that L1 inhibition by **2** does not occur to a significant extent.

As, to date, the inhibition of subclass B3 MBLs by bicyclic boronates has not been reported, we investigated the inhibition of purified L1 and L2 by the three β-lactamase inhibitors using the fluorogenic β-lactamase substrate FC5 as a new reporter for L1 and L2 (192). Calculated *k*cat/K_M values clearly demonstrate that FC5 is hydrolysed with a higher efficiency than other β-lactams by both L1 and L2 (**Table 5.1**). IC_{50} measurements revealed that while all three β-lactamase inhibitors inhibit L2 with nanomolar potencies (**Table 5.3**), no inhibition of L1 was observed, even when using inhibitor concentrations up to 2.5 mM. Thus, unlike the case for subclass B1 MBLs (293), the bicyclic boronate **2** is not an effective inhibitor of the subclass B3 MBL L1.

Table 5.3 Inhibition of L2 by β-lactamase inhibitors in vitro

Inhibitor	IC_{50} (nM)	pIC_{50}
Clavulanic Acid	22.3	7.41
Avibactam	14.36	7.84
Bicyclic Boronate **2**	5.25	8.27

5.2.3 Structural basis for inhibition of L2 by avibactam and the bicyclic boronate 2

Collaboration with Dr. Philip Hinchliffe (University of Bristol) allowed data collection, refinement and final structure modelling. The results above demonstrate that, consistent with the effectiveness of β-lactam/β-lactamase inhibitor combinations against *S. maltophilia* strains, L2 is effectively inhibited by both avibactam and the bicyclic boronate **2**. To investigate the molecular basis for this inhibition we crystallised L2 and soaked the crystals in avibactam or **2**. (Consistent with our inhibition kinetics results, we were unable to obtain crystal structures of complexes of L1 with either of these inhibitors). L2 crystallised in the space group $P2_12_12_1$ with two molecules in the

asymmetric unit (**Table 5.4**), and closely conserves the overall SBL fold with, for example, a C alpha RMSD to KPC-2 (PDB 2OV5) of 0.2 Å.

Table 5.4 Data collection and refinement statistics

	Native/(*D*)-glutamate	avibactam	cyclic boronate
Data collection			
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$
Molecules/ASU	2	2	2
Cell dimensions			
a, *b*, *c* (Å)	69.68, 84.72, 93.71	69.49, 84.31, 93.67	70.38, 85.69, 113.18
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Wavelength(s) (Å)	0.97625	0.97949	0.92819
Resolution (Å)*	55.92 – 1.19 (1.21 – 1.19)	53.62 – 1.35 (1.37 – 1.35)	44.10 – 2.09 (2.14 – 2.09)
R_{pim}	0.050 (0.778)	0.053 (0.806)	0.126 (0.800)
$CC_{1/2}$	0.999 (0.625)	0.998 (0.616)	0.992 (0.534)
I / σ(I)	10.7 (1.9)	9.8 (1.3)	8.0 (1.4)
Completeness (%)	99.7 (98.3)	96.8 (94.2)	99.3 (99.5)
Redundancy	8.6 (8.0)	11.5 (9.9)	13.1 (13.3)
Refinement			
Resolution (Å)	55.92 – 1.19	53.62 – 1.35	42.845 – 2.09
No. reflections	176,092	116,868	40,861
R_{work} / R_{free}	0.1522 / 0.1640	0.1618 / 0.1781	0.1756 / 0.2143
No. atoms			
Protein	8180	3478	4035
Solvent	763	644	335
Ligand	34	56	25
B-factors (Å^2)			
Protein	15.9	17.3	28.8
Solvent	29.3	34.5	41.2
Ligand	24.3[1]	22.45[2]	29.9[3]
R.m.s. deviations			
Bond lengths (Å)	0.007	0.011	0.010
Bond angles (°)	0.963	1.349	1.249
Ramachandran (%)			
Outliers	0.00	0.00	0.00
Favoured	97.38	97.36	97.16

*Values in parentheses are for highest-resolution shell.
[1]Chain A B-factor 18.4; chain B B-factor 30.3
[2]Chain A B-factor 22.9; chain B B-factor 22.0
[3]Only present in chain B.

L2 crystals formed in a reagent containing racemic mixture of the amino acids glutamate, alanine, lysine and serine. The active site manifests clear *Fo-Fc* density into which a molecule of *D*-glutamate could be modelled (**Figure 5.2**), indicating the *D*-enantiomer preferentially binds to L2. However, binding does not perturb the active site conformation compared with an un-complexed L2 crystal structure (PDB 1O7E) (**Figure 5.3**), preserving positioning of the hydrolytic (deacylating) water with respect to

Glu166, Asn170 and Ser70 (see **Table 5.5** for distances), and the conformation of the conserved, catalytically important Lys73 (326-328).

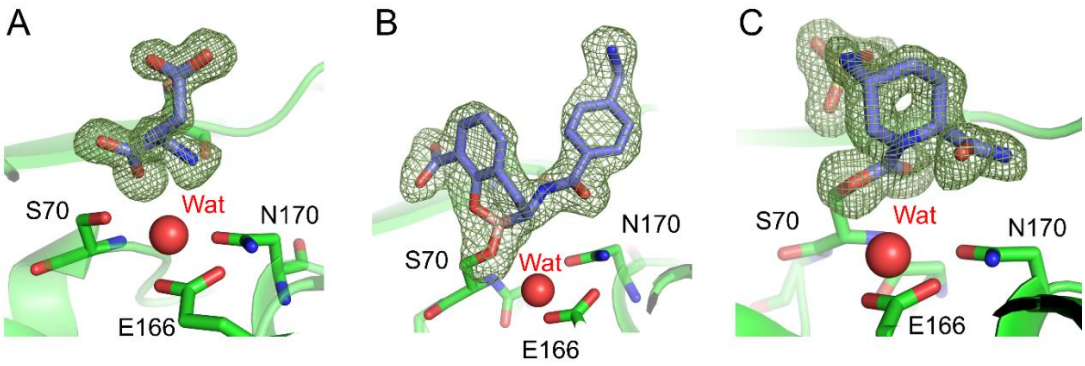


Figure 5.2 L2 active site views showing electron density maps calculated after removal of ligand

F_o-F_c density (green, contoured at 3σ) calculated from the final model after removal of (A) *D*-glutamate, (B) Boronate 2 and (C) avibactam. Residues coordinating the 'hydrolytic' water (red sphere, 'Wat') are shown as sticks and labelled (Ser70, Glu166 and Asn170).

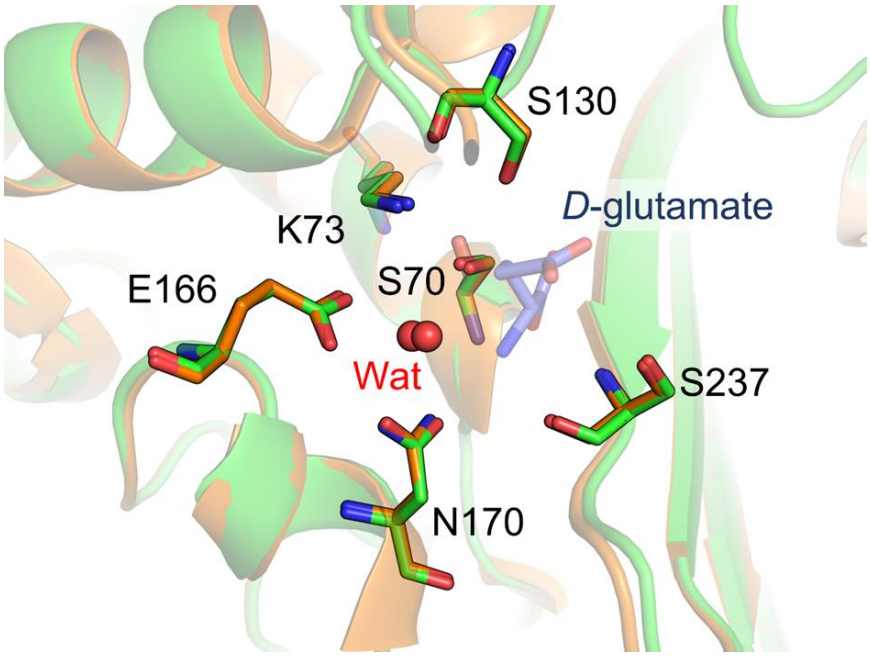


Figure 5.3 Comparison of the active sites of L2:*D*-glutamate with L2 native

L2:*D*-glutamate (green) and L2 native (PDB 1O7E, orange) are superposed and shown in cartoon, with important catalytic residues shown as sticks and the hydrolytic water (red) as

a sphere.

Table 5.5 Catalytic water interactions (distances in Å) in L2 structures

	Glu166-OE2	Asn170-OD1	Ser70-OG
L2:native (PDB 1O7E)	2.50	2.71	2.71
L2:*D*-glutamate	2.63	2.63	2.55
L2:avibactam	2.55	2.76	2.87
L2:boronate 2	2.49	2.60	2.70

D-glutamate binds non-covalently, through interactions of its carbonyl oxygen with the backbone amides in the oxyanion hole (formed by residues Ser70 and Ser237), the C-terminal oxygen with Ser130-Oγ, and the glutamate amide with the deacylating water (**Figure 5.4A**). Despite these extensive interactions, there is little inhibitory effect, with 100 mM *D*-glutamate reducing L2 activity by just 20% (**Figure 5.5**). *D*-glutamate binds differently compared with the high affinity binding (Ki = 84 pM) of the β-lactamase inhibitory protein (BLIP) to the class A β-lactamase KPC-2 (PDB 3E2K) (329). Interestingly, BLIP binding to KPC-2 involves localisations of an L-aspartate residues at the active site, in a manner related to, but different from, *D*-glutamate binding to L2, and one that does not involve interactions with the oxyanion hole (**Figure 5.6**) (330).

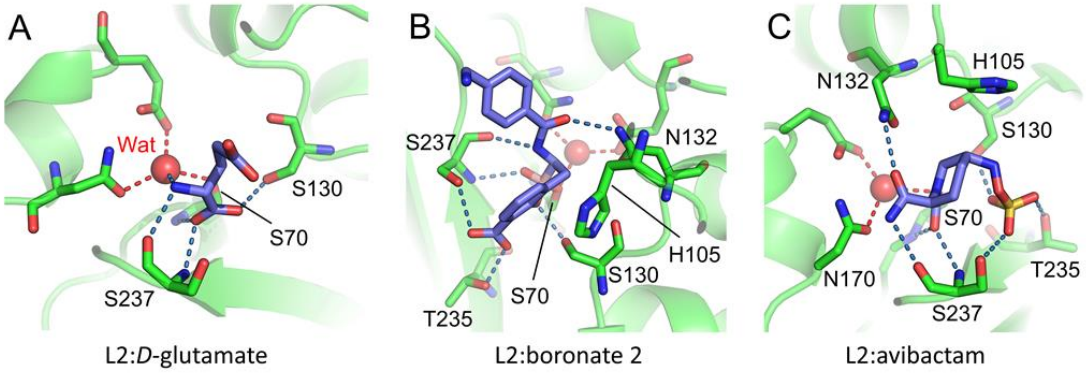


Figure 5.4 Interaction of β-lactamase inhibitors with the L2 active site

View of L2 (shown in green cartoon) active sites with bound ligands (blue sticks), (**A**) *D*-glutamate, (**B**) bicyclic boronate **2** and (**C**) avibactam. Interactions between residues and the catalytic water are shown as red dashes, and interactions between residues and ligand as blue dashes. Labelled residues are those that specifically interact with the ligand.

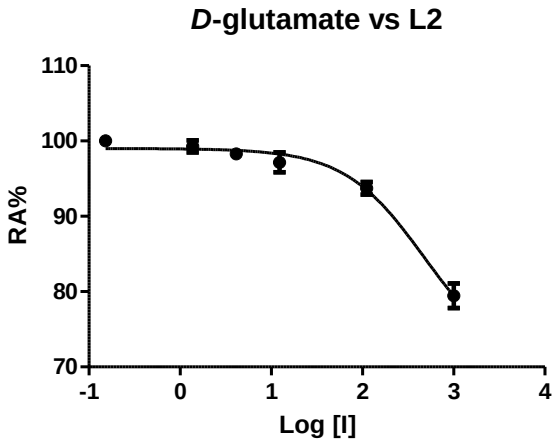


Figure 5.5 Inhibitory effect of *D*-glutamate on L2 catalysis

L2 activity against FC5 reduces by 20% in presence of the highest concentration of *D*-glutamate (100 mM). Figure obtained by fitting residual activity plots using GraphPad Prism.

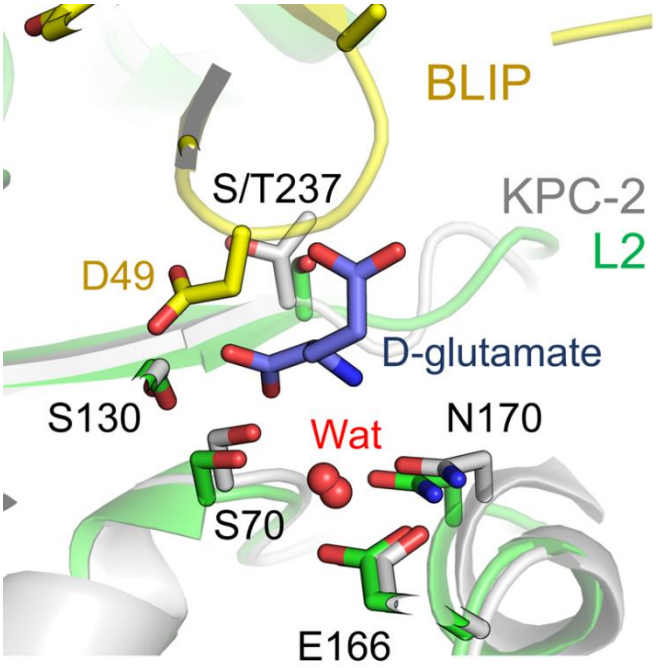


Figure 5.6 Superposition of L2:D-glutamate with KPC-2:BLIP

The L2:*D*-glutamate structure is superposed with the crystal structure (PDB 3E2K) of BLIP (yellow) bound in the active site of KPC-2 (grey). Asp49 (stick, labelled) is the only BLIP residue making interactions in the KPC-2 active site, although binding is significantly different to *D*-glutamate (blue), with the exception of hydrogen bond formation to the side chains of S130 and T237 (labelled sticks; S237 in L2). Residues which interact with the catalytic water (red sphere) or form the oxyanion hole are labelled and shown as sticks.

L2:avibactam and L2:bicyclic boronate **2** complex structures were solved to 1.35 Å and 2.09 Å resolution, respectively, with clear *Fo-Fc* density indicating that both inhibitors react with the active site nucleophile Ser70 to form covalent complexes (**Figure 5.2B and Figure *5.2*C**). Binding by both compounds reveals no significant changes in the L2 active site in comparison with the apo or *D*-glutamate structures. Indeed, in both structures the deacylating water is positioned similarly to the native and *D*-glutamate-bound structures (**Table 5.5**).

The bicyclic boronate **2** binds L2 (**Figure 5.4B**) with the boron atom clearly in a tetrahedral geometry, as observed previously on binding of the cyclic boronate **1** to CTX-M-15 (another class A ESBL) (310) and OXA-10 (a class D SBL) (293), mimicking the first tetrahedral intermediate formed during β-lactam hydrolysis. As in *D*-glutamate

binding, the assigned OH group on the boron atom is positioned to make strong interactions with the backbone amides of Ser70 (2.95 Å) and Ser237 (3.1 Å) in the oxyanion hole. The bicyclic boronate **2** makes additional hydrogen bonds to the side chains of the catalytically important residues Ser130 (2.77 Å to the bicyclic ring oxygen), Asn132 (3.0 Å to the acetamide oxygen), Ser237 (2.96 Å to the carboxylate) Thr235 (2.65 Å to the carboxylate), and the backbone carbonyl oxygen of Ser237 (3.1 Å to the acetamide nitrogen). In addition, binding is stabilised by significant hydrophobic interactions with His105.

Avibactam (**Figure 5.4C**) binds to L2 in its ring opened form, forming a carbamoyl-enzyme complex (300) in which its six-membered ring is in a chair conformation, a conserved feature in other structurally characterised avibactam:β-lactamase complexes (113, 331-335). Highlighting the importance of the oxyanion hole, as with both *D*-glutamate and the bicyclic boronate **2**, the avibactam derived carbamoyl oxygen is positioned to make hydrogen bonds with the oxyanion hole backbone amides of Ser70 (2.75 Å) and Ser237 (2.85 Å). His105 is also involved in providing stabilising hydrophobic interactions (3.49 Å), while the carbamoyl NH interacts with the backbone carbonyl of Ser237 (3.08 Å) and the Asn132 sidechain (2.97 Å). The carbamoyl NH interactions may be relatively less important as they present in only one molecule in the asymmetric unit (chain B). The avibactam sulfate moiety interacts with the OH groups of both Thr235 (3.10 Å) and Ser130 (2.88 Å), with an additional 3.19 Å interaction with Ser237 in chain B.

Our structural data reveal that the bicyclic boronate **2** binds to the ESBL L2 in a manner similar to that previously observed for the closely related bicyclic boronate **1** binding to the ESBL CTX-M-15. For the bicyclic boronate **2**, binding of the tetrahedral boron atom to L2 and conformation of the bicyclic fused core are all consistent with the CTX-M-15:bicyclic boronate **1** structure (310); there is only slight variation in the amide/benzamide side chain conformations (**Figure 5.7A**). Furthermore, formation of the L2 carbamoyl-enzyme complex by avibactam results in a conformationally similar mode of binding compared with structurally-characterised complexes with the class A SBLs KPC-2 (PDB 4ZBE) (331), SHV-1 (PDB 4ZAM) (331) and CTX-M-15 (PDB 4S2I) (332) (**Figure 5.7B**), and is consistent with data indicating avibactam to be similarly effective against these enzymes (300, 331, 336) However, some subtle differences in active-site interactions are observed (**Figure 5.8**). In particular, while the avibactam carbamoyl hydrogen bond with Asn132 is conserved, the weaker carbamoyl interaction with the carbonyl oxygen of L2-Ser237 is not, highlighting that such an interaction is not essential for binding (337). Furthermore, the avibactam sulfate moiety interaction

with Thr235 is likely important as it presents in all SBLs, while interaction with Ser130 is present in KPC-2 alone. In SHV-1:avibactam, interaction of the sulfate group with the non-conserved Arg244 essentially substitutes for the sulfate-Ser237 interaction in other SBLs (Thr237 in KPC-2). In both SHV-1:avibactam and KPC-2:avibactam the 'hydrolytic' deacylating water molecule hydrogen bonds to the avibactam carbamoyl, while this interaction is not observed with either CTX-M-15:avibactam or L2:avibactam. The avibactam-derived sulfate-bonded nitrogen is in the same conformation as in KPC-2/SHV-1:avibactam and, unlike in the CTX-M-15:avibactam complex, is directed away from the six-membered ring and distant from Ser130 (Ser130, 3.57 Å) (332) and consequently is not primed for re-cyclization (304) in which this residue is involved (**Figure 5.7B**). The CTX-M-15:avibactam complex therefore remains to date as the only crystallographic evidence for avibactam reacting with an SBL in a conformation ideal for re-cyclization (332). Thus, the rate at which the avibactam derived complex can re-cyclize to reform intact avibactam may vary between SBLs.

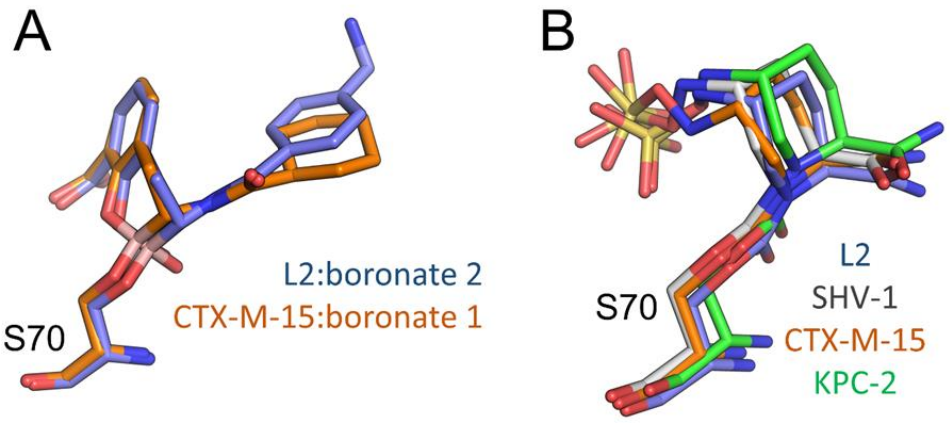


Figure 5.7 Bicyclic boronate and avibactam binding conformations in Class A β-lactamases

Superposition of inhibitors, shown as sticks, bound in the active sites of Class A β-lactamases. (A) Bicyclic boronates bound to L2 (blue, bicyclic boronate **2**) and CTX-M-15 (grey, bicyclic boronate **1**). (B) Avibactam bound to L2 (blue), SHV-1 (grey), KPC-2 (green) and CTX-M-15 (orange). Note the common binding mode for the bicyclic boronate bicyclic core and most of the avibactam structure; there are differences in the orientations of the avibactam core derived nitrogen (see text).

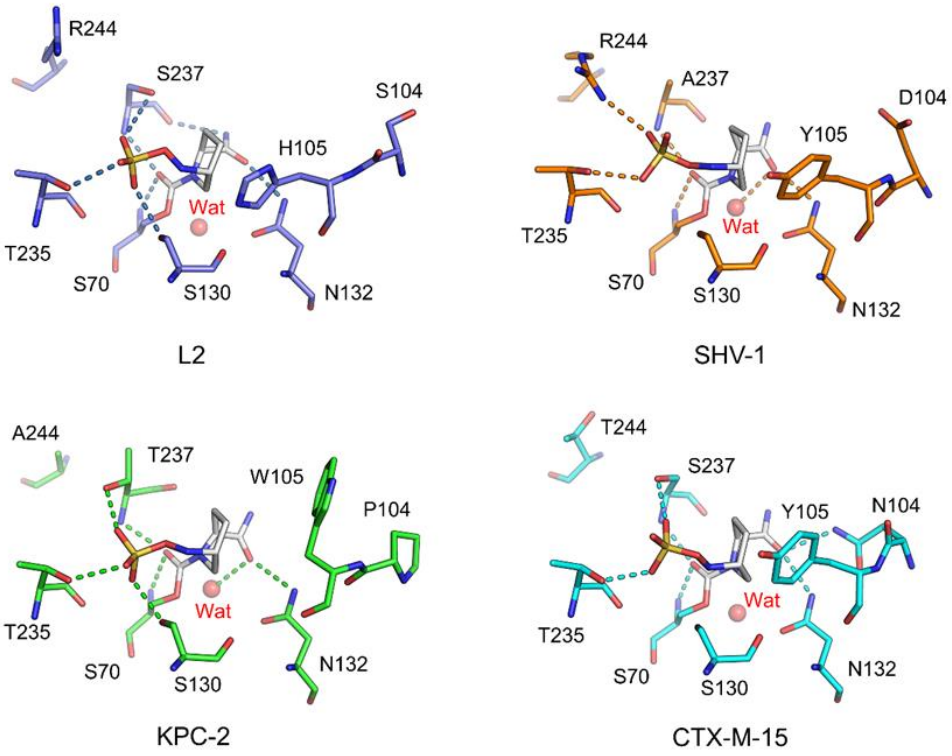


Figure 5.8 Comparisons of structurally characterised modes of binding of avibactam in class A SBLs

Avibactam (grey sticks) is shown as complexed with L2 (blue, this study), SHV-1 (orange, PDB 4ZAM), KPC-2 (green, PDB 4ZBE), and CTX-M-15 (cyan, PDB 4S2I).

5.2.4 β-lactamase production is not induced by avibactam and the bicyclic boronate 2

One important consideration when deploying β-lactamase inhibitors into clinical practice is that some can interact with penicillin binding proteins and trigger β-lactamase induction pathways carried by many bacteria (110). L1 and L2 production in *S. maltophilia* is controlled by a transcriptional regulator, AmpR, which is responsive to β-lactam challenge via sensing β-lactam mediated perturbations in peptidoglycan breakdown and recycling (215, 338). Hence we tested the ability of β-lactamase inhibitors to induce β-lactamase production in *S. maltophilia*. Clavulanic acid induces L1 production (measured using meropenem hydrolysis in cell extracts) at a similar level to the positive control β-lactam cefoxitin in the *S. maltophilia* wild type strain K279a (**Figure 5.9**). This observation rationalizes why clavulanic acid does not reduce the MIC of ceftazidime against *S. maltophilia* K279a (**Table 5.2**) induction of L1 (**Figure**

5.9) overcomes inhibition of L2 (**Table 5.3**) because L1 can hydrolyse ceftazidime (**Table 5.1**). Since L1 does not hydrolyse aztreonam (**Table 5.1**), however, clavulanic acid reduces the aztreonam MIC against K279a, despite its ability to induce L1 production (**Table 5.2**, **Figure 5.9**). Notably, by contrast with clavulanic acid, both avibactam and the bicyclic boronate **2** reduce ceftazidime MICs against K279a (**Table 5.2**). This observation is explained by the important finding that neither avibactam nor **2** induces L1 to any measurable extent (**Figure 5.9**), yet both inhibit L2 (**Table 5.3**).

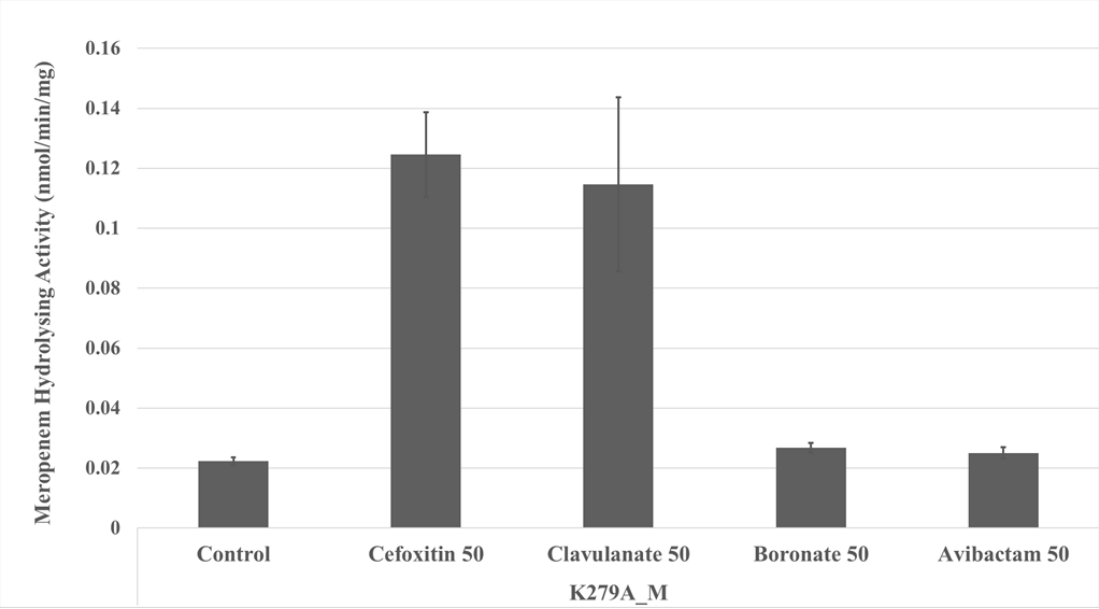


Figure 5.9 L1 β-lactamase induction by β-lactamase inhibitors in *S. maltophilia* K279a

S. maltophilia isolate K279a was incubated in presence of different potential inducers (cefoxitin, clavulanic acid, the bicyclic boronate 2, or avibactam) at 50 mg/L). L1 activity was measured from the cell extracts in a 96-well plate reader by determining meropenem hydrolysis (100 μM) at λ=300 nm. Protein concentration was determined by using the BioRad protein assay dye reagent. Specific activity was calculated by using the extinction coefficient of 9600 AU/M/cm and a pathlength correction for the microplate (0.62 mm). Data presented are means +/- SEM, n=3.

5.2.5 Selection and characterisation of mutants which overcome the reversal of ceftazidime resistance by avibactam and the bicyclic boronate 2

Avibactam is currently only clinically available in combination with ceftazidime. The fact that L1 induction by clavulanic acid overcomes its ability to reduce ceftazidime MICs against *S. maltophilia* (**Figure 5.9, Table 5.2**) led us to suggest that L1/L2 hyper-producing, ceftazidime resistant strains might further mutate to be ceftazidime resistant in the presence of avibactam and the bicyclic boronate **2** by producing even more L1. To investigate this possibility, we used a K279a *ampR* mutant, M11, which is ceftazidime resistant due to L1/L2 hyper-production but where ceftazidime resistance can be reversed following treatment with avibactam or **2** at 10 mg/L (**Table 5.6**). We aimed to identify mutants able to grow on ceftazidime at >32 mg/L (i.e. clinically resistant, according to CLSI breakpoints (325) in the presence of either avibactam or **2** at 10 mg/L. Mutants were readily obtained; those selected using ceftazidime/avibactam were also resistant to ceftazidime/**2**, and *vice versa* (**Table 5.6**). To investigate the basis for resistance, LC-MS/MS proteomics was used to quantify changes in protein production in the two mutants. In both cases, L1 was produced at levels ~3-fold greater than in the parent strain (**Annex 4, Figure 5.10A**). This result was confirmed by measuring L1 enzyme activity in cell extracts using meropenem as substrate (**Figure 5.10B**). Thus, hyper-production of L1 can overcome the ability of these L2-specific inhibitors to rescue ceftazidime activity against a ceftazidime-resistant strain. Importantly, however, the mutants were still sensitive to the aztreonam/avibactam or aztreonam/**2** combinations (**Table 5.6**) as L1 cannot hydrolyse aztreonam (**Table 5.1**). The L1 hyper-producing phenotype, blocking reversal of ceftazidime, but not aztreonam, resistance by β-lactamase inhibitors is clearly relevant, because it is displayed by clinical isolate CI-31 (**Table 5.2**). WGS analysis of the M11 derivatives showed variations in *smlt0009* but siderophore production was similar to the K279a (data not shown). Despite our efforts, we could not identify a responsible mutation for L1 production.

Table 5.6 MICs (mg/L) of β-lactams against *S. maltophilia* mutants in the presence of β-lactamase inhibitor (10 mg/L)

	Ceftazidime			Aztreonam		
	-	+BOR	+AVI	-	+BOR	+AVI
K279a	4	0.5	1	128	1	1
M11	128	8	2	256	1	1
MA27	256	32	32	256	4	4
MB25	256	64	128	256	4	4

Shaded values represent resistance according to CLSI breakpoints.

BOR, bicyclic boronate **2**; AVI, avibactam.

MA27 and MB25 were selected for growth at 32 mg/L ceftazidime in the presence of 10 mg/L avibactam or bicyclic boronate **2**, respectively using M11 as parent strain. M11 is an L1/L2 hyper-producing mutant derived from K279a, which is wild-type (325).

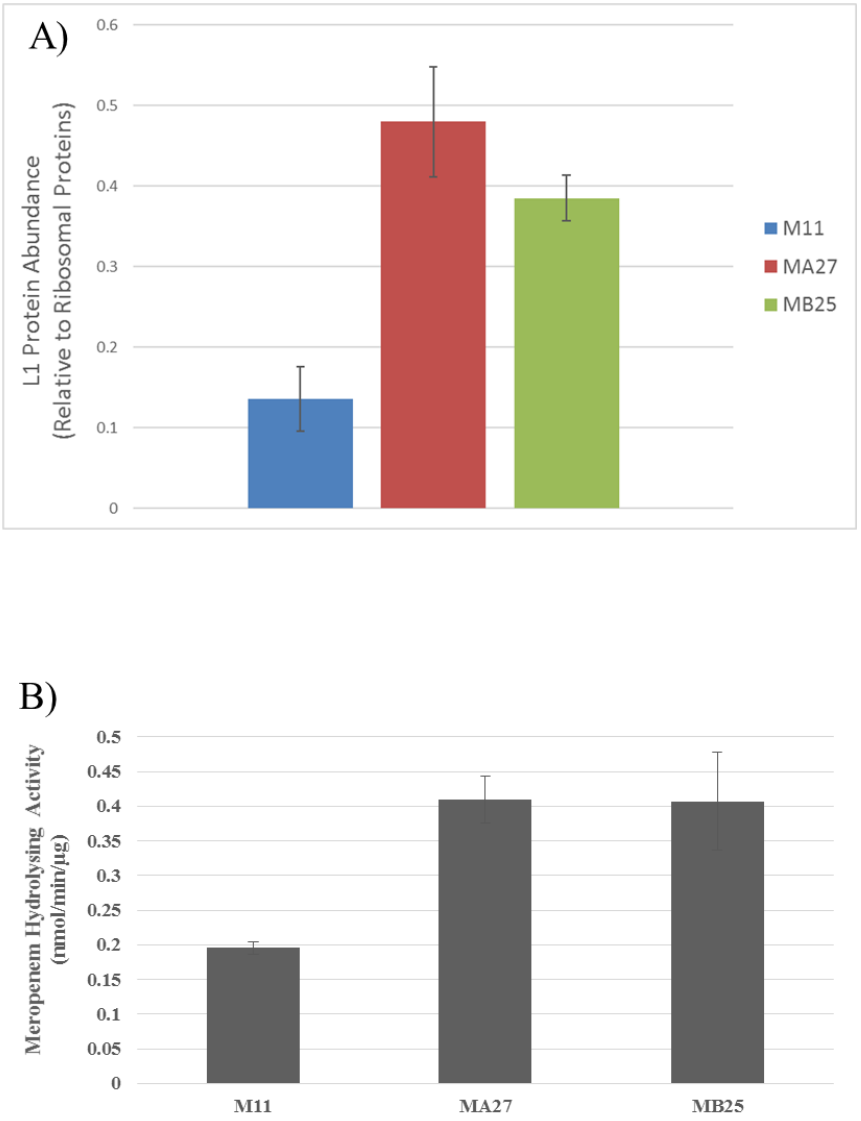


Figure 5.10 L1 activity of Inhibitor Resistant Mutants

In (A), L1 protein abundance data (relative to mean ribosomal protein abundance for each sample). Full proteomics data are shown in (272). In (B), L1 enzyme activity in cell extracts is reported as meropenem hydrolysis rate. Data are reported as mean +/- SEM, n=3 for the parent strain, M11, and the two mutants (MB25 and MA27), which are resistant to ceftazidime/avibactam and ceftazidime/**2**.

Even though our structural and kinetic work confirm that L2 is potently inhibited by avibactam and the bicyclic boronate **2**, we predicted failure of avibactam/ceftazidime against *S. maltophilia*. Our prediction was based on the observation that L1-hyper-producing mutants are readily obtained from *S. maltophilia*

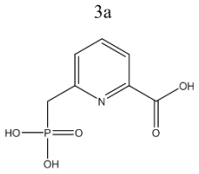
isolates (**Figure 5.10**), and avibactam does not inhibit MBLs (305) . Whilst **2** inhibits subclass B1 MBLs (293), our work reveals that it does not inhibit the subclass B3 MBL, L1 (**Table 5.3**) and so **2**/ceftazidime also overcome by L1 hyper-production (**Table 5.6, Figure 5.10**). It may be possible to modify **2** and so generate a broader-spectrum MBL inhibitor. However, a key finding of this work is that such a modification might not be essential. Avibactam and **2** both facilitate killing of *S. maltophilia* when paired with the monobactam, aztreonam, reducing MICs to ≤ 4 mg/L even in the pan-resistant clinical *S. maltophilia* isolate, CI-31 (**Table 5.2**). These data imply that aztreonam/avibactam and aztreonam/**2** may have a promising clinical future for treatment of infections caused by this most intractable of species. The fact that efflux pump over-production does not affect aztreonam/**2** or aztreonam/avibactam activity (**Table 5.2**) gives even greater cause for optimism. It was interesting to read, therefore, a recent clinical case report demonstrating the use of combination therapy with ceftazidime/avibactam plus aztreonam to save the life of a patient with an *S. maltophilia* infection that had failed all prior therapy (339). Our structural, kinetic and whole bacterial killing data would lead to the conclusion that ceftazidime was probably superfluous in this success, but our work indicates that ceftazidime/avibactam plus aztreonam might be routinely considered in the clinic for use against seemingly untreatable *S. maltophilia* infections whilst aztreonam/avibactam works its way through the clinical trials system. Positive results have been seen with ceftazidime/avibactam plus aztreonam against Enterobacteriaceae isolates carrying multiple β-lactamases, but the combination was not universally efficacious (340).

In many respects, because of its inability to inhibit L1, the bicyclic boronate **2** acts against *S. maltophilia* very similarly to avibactam. One potentially significant difference is that avibactam is hydrolysed by L1, but **2** retains its structural integrity. This hydrolysis is slow (272), and even if L1 is hyper-produced, it is not significant enough to confer aztreonam/avibactam resistance (**Table 5.6**). However, there is a chance that L1 mutants might be selected with greater avibactam hydrolytic activity, reducing the degree of L2 inhibition and raising the MIC of aztreonam/avibactam into the resistant range. This observation may also be of relevance to other avibactam-like compounds in development, e.g. Relebactam (341). In contrast, given that modification of **2** in the presence of wild-type L1 is undetectably slow (272), evolution to increased breakdown may require significantly more steps, potentially increasing the long-term efficacy of aztreonam/**2** as a combination to treat *S. maltophilia* infections.

5.2.6 Potentiation of Meropenem activity in presence of metallo-β-lactamase inhibitors

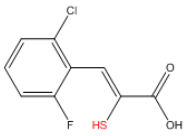
Apart from the aztreonam/**2** combination, we explored other alternatives to counteract the β-lactamase activity of L1 in *S. maltophilia*. Here, we tested the simplest of a series of 6-phosphonomethylpyridine-2-carboxylate (PMPC) derivatives which contains a pyridine core and a phosphonomethyl group at position C6, inhibitor **3a**. Initially, uptake of **3a**, was tested against K AMI 32, the SmeYZ overproducer, and a K279a:*smeJKWZP*$^{-}$, lacking efflux pumps SmeJ/K/W/Z/P (173). Meropenem MIC values showed that increased efflux does not affect activity of **3a**. Originally meropenem MIC values for both strains are equal given the intrinsic error on the test which is two-fold (342). Despite our finding that high concentrations of **3a** are needed to reverse meropenem resistance (**Table 5.7**) in *S. maltophilia*, data here obtained present **3a** as an interesting scaffold structure on which to generate more potent broad-spectrum MBL inhibitors.

Table 5.7 Meropenem activity against *S. maltophilia* isolates in presence of PMPC (3a)

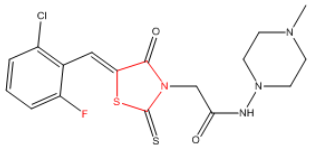
		Meropenem MIC (mg.L^{-1})				
Strain	**Resistance determinant**		3a 			
		in absence of inhibitor	**in the presence of PMPC (3a) at stated concentration (mg.L^{-1})**			
			16	**32**	**64**	**128**
K279a	Basal L1, L2	**16**	16	16	8	4
K AMI 32	Overexpression SmeYZ	**16**	16	16	8	8
K279a: JKWZP $^{-}$	SmeJ,K,W,Z,P Knocked out	**32**	32	32	16	8

A different approach against L1 activity was the use of rhodanine based derivatives (provided by University of Oxford). Here, we tested the previously reported rhodanine (ML302) and its enethiol fragment (ML302F) (317), as well as the rhodanine analogue (210) and the ML302F analogue (310). Meropenem antimicrobial activity was tested in presence of the four inhibitors (10mg.L^{-1}) and against the mutants generated in previous chapters. Interestingly, all four inhibitors restore meropenem susceptibility in the wild-type strain, some clinical isolates, (CI-29, CI-31, CI-20, CI-24), β-lactamase hyper-producing strains (KCAZ10, KCAZ14, KM11), efflux-pump overproducers (KAMI32 and KMOX8). In the extensively-drug resistant CI-31, only the rhodanine analogue 210 is able to restore meropenem susceptibility while in the avibactam/ceftazidime and boronate **2**/ceftazidime resistant mutants L1 hyper-producing mutants (MA27 and MB25, described above) not even 210 restores susceptibility (**Table 5.8**).

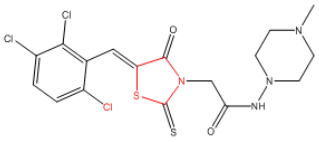
Table 5.8 MIC obtained for meropenem in the presence and absence of rhodanine derivatives



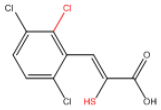
310



210



ML302



ML302F

	Resistance	Meropenem MIC mg.L^{-1}				
		in absence of inhibitor	in presence of the inhibitor 10mg.L^{-1}			
			310	210	M302F	M302
K279a	Basal L1,L2	**16**	0.25	0.25	0.5	0.5
CI-29		**32**	0.25	0.25	0.5	0.25
CI-31	IS *ampDI*	**256**	4	2	4	4
CI-20		**16**	0.25	0.25	0.25	0.25
CI-24		**2**	1	1	1	1
KCAZ10	159-168del in AmpD	**128**	1	1	1	2
KCAZ14	140-146del in Mpl	**64**	1	1	2	2
KAMI32	Overexpression SmeYZ	**8**	0.25	0.5	0.5	0.5
KMOX8	Overexpression SmeDEF	**8**	0.25	0.5	1	1
K M11	Asp135Asn in AmpR	**128**	2	1	2	2
MB25	L1 overexpression	**256**	8	4	8	8
MA27	L1 overexpression	**256**	16	4	8	8

Shaded values indicate clinically relevant resistance according to CLSI breakpoints (325)

5.3 Conclusion

In conclusion, our combined results reveal the potential of non-classical non β-lactam containing β-lactamase inhibitors, including the clinically approved compound avibactam, and the cyclic boronates (some of which are presently in clinical trials) for treatment of *S. maltophilia*, particularly when partnered with the monobactam aztreonam, and perhaps other aztreonam-like β-lactams currently in development (343). Given the structural differences between avibactam, cyclic boronates, and the β-lactam based inhibitors, it would seem that there is considerable scope for the identification of new types of β-lactamase inhibitors of potential clinical utility against Gram-negative bacterial pathogens. In fact, within that scope we have managed to identify the potential benefits of two compounds, 6-phosphonomethylpyridine-2-carboxylates and rhodanine based inhibitors, as L1 β-lactamase inhibitors (316, 318). However, results here obtained highlight the ability of *S. maltophilia* to acquire resistance mechanisms to overcome inhibitor activity which makes of it an interesting model to study in the search of a pan β-lactamase inhibitor that protects our current antibiotics.

6 Characterisation of sideromimic modified lactivicin against *S. maltophilia*

6.1 Introduction

Lactivicin is highly unusual in that it is the only non β-lactam natural product known to target penicillin binding proteins (PBPs). Unlike the β-lactams, which remain the most important antimicrobial class, Lactivicin contains cycloserine and γ-lactam motifs, but like the β-lactams, Lactivicin reacts covalently with PBPs to form a stable acyl-enzyme complex (344) (**Figure 6.1A Figure *6.1*B**). However, Lactivicin has poor penetration into Gram-negative bacteria and is susceptible to at least some β-lactamase enzymes (345-347). A deeper understanding of the interactions between Lactivicin and its derivatives and their various enzyme targets has led to the rational design of synthetic derivatives with higher potency against bacteria and reduced susceptibility to β-lactamases (190, 344, 348). Recently, it has been shown that sideromimic modification of the synthetic Lactivicin compound 13 (hereafter referred to as LTV13)(347) results in more favourable IC_{50} values against *P. aeruginosa* PBPs and improved penetration into *P. aeruginosa* strain PA01 via interaction with the siderophore receptors and uptake systems of this strain. One of these Lactivicin derivatives, the phthalimide-conjugated compound 17 (hereafter referred to as LTV17; **Figure 6.1C**), seems particularly promising (190).

This chapter describes the activity of Lactivicin derivatives against *S. maltophilia*. We aimed to test lactivicin derivatives against extensively drug resistant clinical *S. maltophilia* isolates and characterise acquired resistance to these compounds.

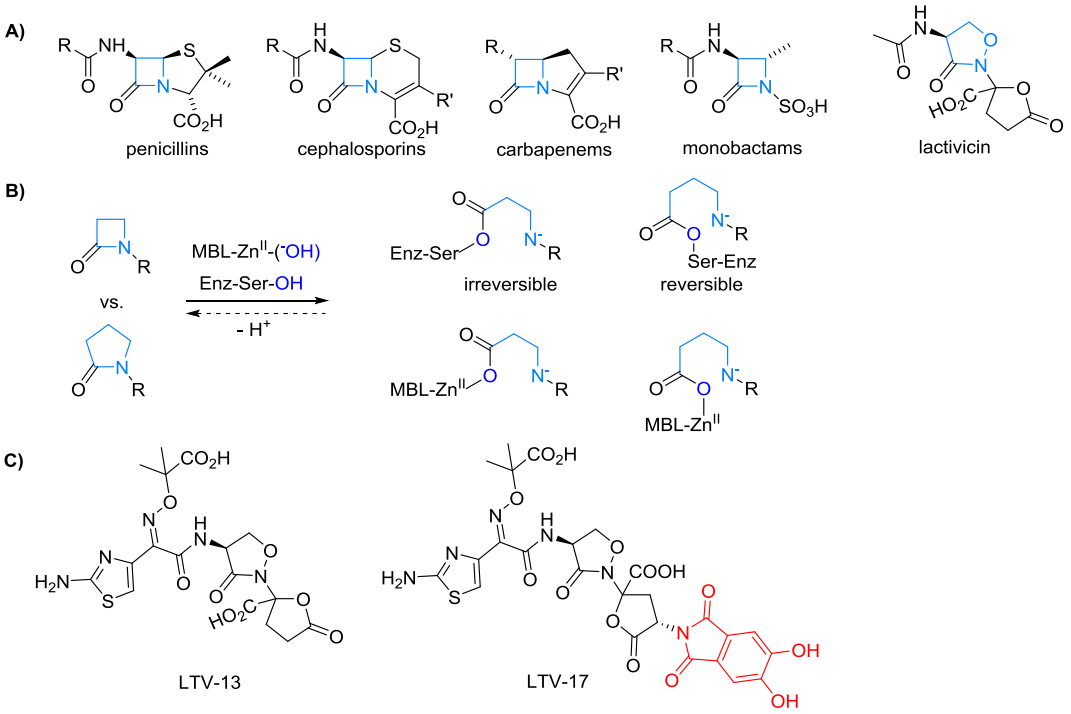


Figure 6.1 Structures and mechanism of action of β- and γ-lactams

A) Structures of β-lactam antibiotics and γ-lactam Lactivicin; B) Outline of mechanism of action of β- and γ-lactams; C) Structures of LTV13 and LTV17 used in the current study.

6.2 Results

6.2.1 MICs of Lactivicin derivatives against *S. maltophilia* clinical isolates

MICs for LTV13 and LTV17 were determined against a selection of clinical isolates representing key Gram-negative species where multi-drug resistance is frequently a problem. These data confirmed the potential of LTV17 and its MIC against the multi-drug resistant *S. maltophilia* isolate K279a (183) is remarkably low, and more than 1000 fold more potent than LTV13 (**Table 6.1**).

Table 6.1 MICs of Lactivicin derivatives against clinical Gram negative isolates

Isolate	**LTV13 MIC (μg.mL^{-1})**	**LTV17 MIC (μg.mL^{-1})**
Klebsiella pneumoniae NCTC5055	2	0.5
Citrobacter freundii D571	512	16
Enterobacter cloacae 30950	>512	16
Enterobacter aerogenes 15-8358A	64	4
Serratia marcescens NCTC1041	4	4
Pseudomonas aeruginosa NCTC10662	256	1
Stenotrophomonas maltophilia K279a	64	≤0.0625
Acinetobacter baumannii 39-4034C	>256	8

In **Chapter 3,** we had established the ceftazidime susceptibility of the world-wide collection of *S. maltophilia* clinical isolates whilst in **Chapter 4** levofloxacin and minocycline susceptibilities were evaluated. Here we expanded the antibiotic susceptibility profile by adding trimethoprim-sulfamethoxazole (SXT), chloramphenicol and ticarcilllin-clavulanic acid to the survey. 54% of the 50 isolates were susceptible to SXT, 18% was susceptible to ticarcillin-clavulanic acid and 38% to chloramphenicol.

Initially, the isolates were divided into two groups: 23/50 that are STX resistant and 27/50 that are STX sensitive. The two groups were then sub-divided based on how many alternative antimicrobials they remain sensitive to. Finally, the MICs of LTV13 and LTV17 were determined against the 50 isolates using standard CLSI broth micro-dilution methodology (**Table 6.2**). Clearly, LTV17 is very potent against all of these *S. maltophilia* clinical isolates, including extensively drug resistant strains. The highest MIC seen was 0.25 μg.mL^{-1}, and the MIC90 for the 50 isolates was 0.063 μg.mL^{-1}.

Table 6.2 MICs of Lactivicin derivatives against *S. maltophilia* clinical isolates having different profiles of resistance to last resort antibiotics.

Resistance phenotype	**Number of isolates where LTV13 MIC (μg.ml^{-1}) is:**					**Number of isolates where LTV17 MIC (μg.ml^{-1}) is:**			
	16	**32**	**64**	**128**	**256**	**≤0.031**	**0.063**	**0.125**	**0.25**
SXT^{R}. Sensitive to 0 to 2 alternatives	0	0	3	5	1	5	3	0	1
SXT^{R}. Sensitive to 3 to 5 alternatives	1	0	7	6	0	13	1	0	0
SXT^{S}. Sensitive to 1 to 3 alternatives	0	0	4	9	1	8	3	3	0
SXT^{S}. Sensitive to 4 or 5 alternatives	0	5	3	5	0	12	1	0	0

6.2.2 MICs of Lactivicin derivatives against *S. maltophilia* clinical isolates

One of the reasons for the initial failure of Lactivicin and its early derivatives as an antibiotic was susceptibility to β-lactamase enzymes, coupled with an ability to induce β-lactamase production in bacteria where inducible enzymes exist (345-347). As discussed in chapter 3 *S. maltophilia* has two inducible β-lactamases, L1 and L2, which are co-ordinately controlled by an AmpR type transcriptional regulator (160). Genetic disruption of one of the main targets of Lactivicin, PBP1A has been shown to constitutively activate β-lactamase production in *S. maltophilia* (214, 232). As expected, therefore, we found that treatment of two well characterised *S. maltophilia* clinical isolates, K279a and N531 (183), with LTV13 or LTV17 induced β-lactamase production to a similar extent as the β-lactam antibiotics cefoxitin and imipenem when added to growing cells at concentrations proportionate to the compounds' relative MICs (**Figure 6.2**).

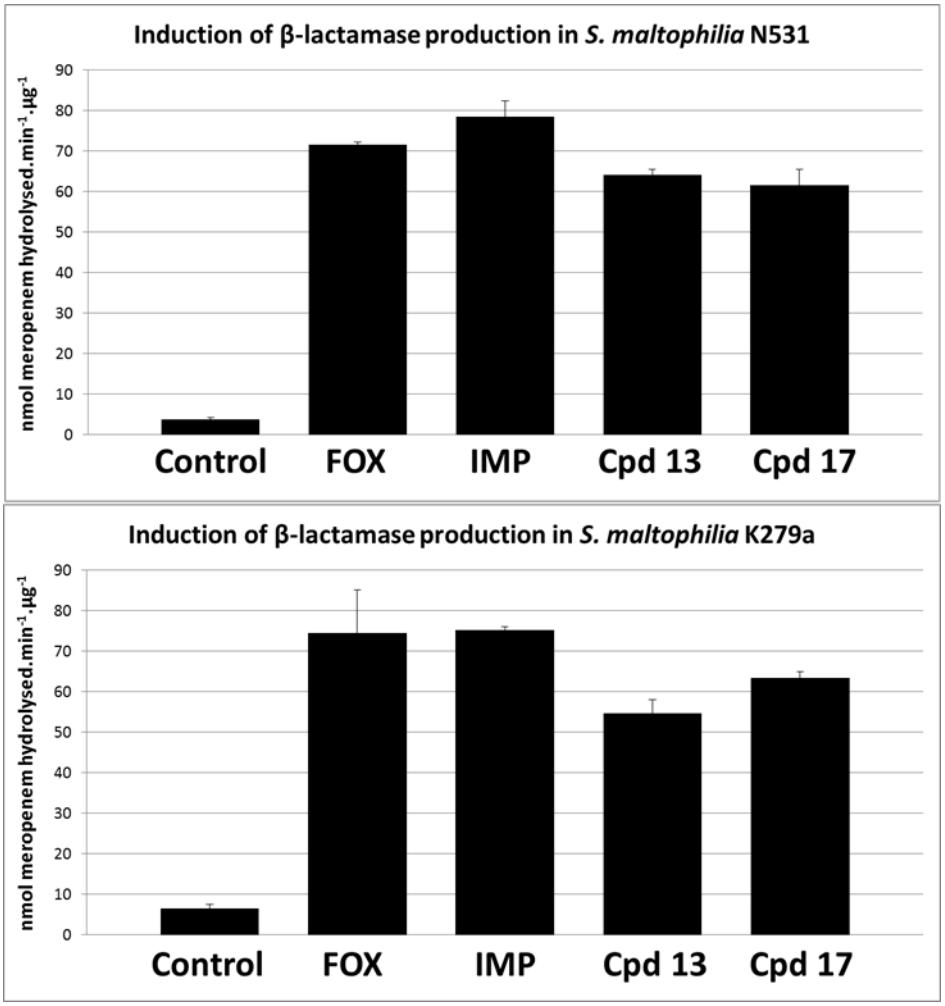


Figure 6.2 Effect of β-lactams and Lactivicin derivatives on the production of β-lactamase by *S. maltophilia* clinical isolates

Bacteria were incubated in the presence of inducer (100 µg.mL^{-1} cefoxitin, 10 µg.mL^{-1} imipenem, 50 µg.mL^{-1} LTV13 and 0.35 µg.mL^{-1} LTV17), cell extracts were prepared and β-lactamase activity in cell extracts was measured as described (22) using 100 µM meropenem as a substrate. Protein concentration was determined using the BioRad protein assay dye reagent concentrate, and an extinction coefficient of 9600 AU/M/cm for meropenem was used to calculate the specific meropenem hydrolysing activity in each cell extract. Data presented are means +/- standard error of the mean, n=3.

6.2.3 Breakdown of Lactivicin derivatives by *S. maltophilia* β-lactamases

This work was performed by our collaborators in Oxford. Since LTV13 and LTV17 can both strongly induce L1 and L2 β-lactamase production, one explanation for

the increased potency of LTV17 versus LTV13 is that LTV17 is not such a good substrate as LTV13 for the *S. maltophilia* β-lactamases. To test this hypothesis, NMR spectroscopy was used to evaluate the time-dependent hydrolysis of LTV13 and LTV17 by purified recombinant L1 and L2 β-lactamases. L2 was able to totally hydrolyse 400 µM ampicillin in less than 5 min (- data not shown), whilst ~95% of the LTVs remained intact. Longer incubation times confirmed that the Lactivicin derivatives are not substrates for L2. They were both found to be substrates for L1, however, and underwent enzyme catalysed hydrolysis (349). LTV17 was broken down faster than LTV13, however, so reduced susceptibility to L1 does not explain the increased potency of LTV17.

6.2.4 Protection of *S. maltophilia* from Lactivicin derivatives by β-lactamases

Whilst they are clearly substrates, the rate of LTV17 and LTV13 breakdown by L1 was very slow compared with breakdown of meropenem (L1 was able to totally hydrolyse 400 µM meropenem in less than 5 min using similar assay conditions when ~95 and 90% of LTV13 and 17 were still intact – data not shown), so we hypothesised that LTV13 and LTV17 actually kill *S. maltophilia* even though they induce β-lactamase production simply because cellular β-lactamase hydrolysis is too slow to protect the cells. To test this hypothesis, we incubated LTV17 with or without purified L1 and spotted the two mixtures onto a lawn of *S. maltophilia* K279a. In parallel we used meropenem as a control (**Figure 6.3**). Incubation with L1 does not significantly reduce the ability of LTV17 to kill *S. maltophilia* K279a (and also LTV13 – data not shown) where meropenem is rendered totally ineffective by L1. Pre-incubation of LTV17 with L1 for 1 h prior to spotting onto K279a did reduce the zone of clearing, suggestive of modest destruction of LTV17 as shown in the NMR experiments. Importantly, the inhibition zone diameter for LTV17 (and LTV13 – data not shown) is the same against K279a as it is for the *ampR* frameshift mutant derivative K279a::ampR^{FS} which cannot induce β-lactamase production (160), even though the latter is far more sensitive to meropenem (**Figure 6.3**). This confirms that even though L1 can break down LTV17 at a modest rate (349) its induction by LTV17 is not sufficient to protect the cell. This also explains why clinical isolates in our world-wide collection that are known to express β-lactamase constitutively at high levels (11, 22) are no less susceptible to the Lactivicin derivatives than are isolates with normally inducible β-lactamases (**Table 6.2**). Indeed, to confirm this, we tested four L1/L2 hyper-producing mutants previously derived from *S. maltophilia* K279a (160, 214, 350) and found that the MICs of both Lactivicin

derivatives against K279a and these mutants are the same (32 and ≤0.031 μg.mL^{-1} respectively for LTV13 and LTV17).

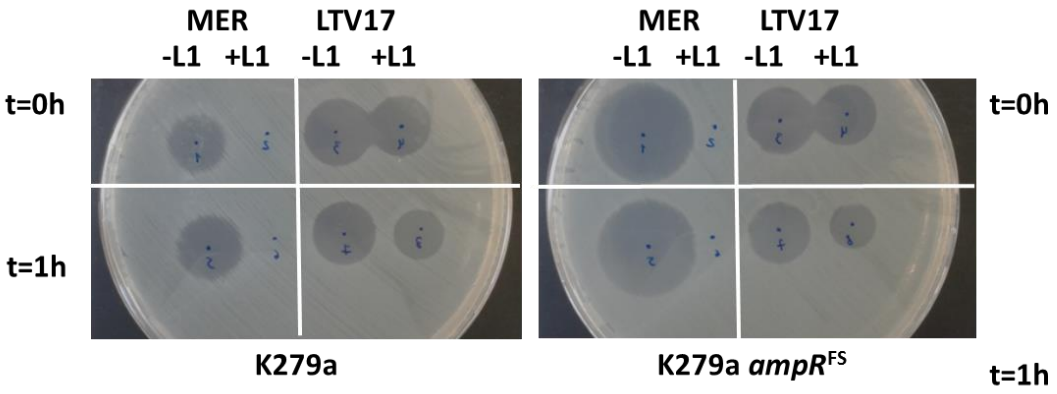


Figure 6.3 Inhibition zone produced by LTV17 and meropenem with and without L1 β-lactamase

A lawn of *S. maltophilia* K279a or the *ampR* frameshift mutant (*ampR*^{FS}) was spread as if for CLSI disc susceptibility testing. In all cases, 2 μL of reaction mixture (50 mM Tris pH 7.5 with meropenem (MER, 12 mM) or LTV17 (0.4 mM) mixed (+L1) or not (-L1) with purified L1 β-lactamase (1 μM final) was immediately spotted onto the lawn (t=0 h) or was spotted following 1 h of pre-incubation at 37°C (t=1 h). The plate was then incubated for 18 h at 37°C and this figure is representative of three biological replicates.

The reason for the dramatically increased potency of LTV17 versus LTV13 against *S. maltophilia* is not due to its relatively weak ability to induce L1/L2 β-lactamase production or its relatively slower hydrolysis by either of these β-lactamases. Both LVT17 and LTV13 are only slowly hydrolysed by L1 β-lactamase, and not at a detectable level by L2, so β-lactamase production by *S. maltophilia* is not actually protective against either Lactivicin derivative. Accordingly, whilst we have not excluded the possibility that there is some increased affinity for its PBP target (s), the 1000-fold increased potency of LTV17 over LTV13 is most likely to be due to an increased rate of entry into *S. maltophilia*. The major difference between LTV13 and LTV17 is the presence of a catechol-type siderophore on the lactone ring of LTV17 (6). Notably, all *S. maltophilia* clinical isolates previously tested, including the K279a isolate used here, exclusively produce catechol-type siderophores (195). Thus it is reasonable to infer that they preferentially take up this type of siderophore, and the antibiotics conjugated to them. Accordingly, it would appear that the siderophore used for LTV17 particularly

favours uptake by *S. maltophilia,* explaining its remarkable potency against this otherwise extensively drug resistant bacterium.

6.2.5 Characterisation of a mutant with reduced susceptibility to lactivicin

A single-step mutant with reduced susceptibility to LTV17 was selected from K279a. The mutant was named K LTV. Initial antimicrobial susceptibility characterisation showed elevated MICs of LTV17 but also ceftazidime. There was no increase in β-lactamase activity (**Table 6.4**) in K LTV leading us to hypothesise that the ceftazidime resistant mutants M1 and M52 identified in chapter 3, would also have reduced susceptibility to LTV17. This proved to be correct **Table 6.3**. When assessing cellular permeability using the Hoescht dye (H33342) accumulation assay, K LTV was actually less permeable even than M1 or M52 (**Figure 6.4**). Similarly to M1 and M52, K LTV whole envelope proteomics showed very similar changes to those observed in M1 and M52 (chapter 3). Specifically, there is downregulation of the TonB energy transducer (Smlt0009) and upregulation of the EntB siderophore biosynthesis enzyme (**Figure 6.5**). siderophore overproduction was confirmed on CAS agar (**Figure 6.6**). WGS confirmed deletion of the proline-rich region in the TonB energy transducer gene (*smlt0009*) (**Figure 6.7**). Accordingly, we conclude that LTV17 uptake is also dependent on a TonB dependent energy transduction system. This is perhaps less surprising than for β-lactams, since siderophore uptake systems are known to be TonB dependent, and it is assumed that the siderophore component of LTV17 is the reason for its rapid uptake. Indeed mutants with reduced susceptibility to other siderophore conjugated antimicrobials have previously been shown to have mutations in siderophore uptake systems (351-353).

Table 6.3 Comparison of MICs (mg/L) values of ceftazidime and lactivin 17 against *S. maltophilia* ceftazidime and lactivicin mutants

	Ceftazidime	LTV17
K279a	4	0.03
M1	256	0.5
M52	256	0.5
K LTV	128	0.25

Shaded values represent reduced susceptibility in reference to the parental strain and in the case of ceftazidime according to CLSI breakpoints

Table 6.4 β-Lactamase activity of lactivicin mutant

Isolate	Mean β-lactamase activity ±SEM
K279a	0.02±0.004
K LTV	0.05±0.005

β-lactamase activity (nmol.min^{-1}.µg^{-1} protein nitrocefin hydrolysis activity in cell extracts) calculated as described in section **2.7**

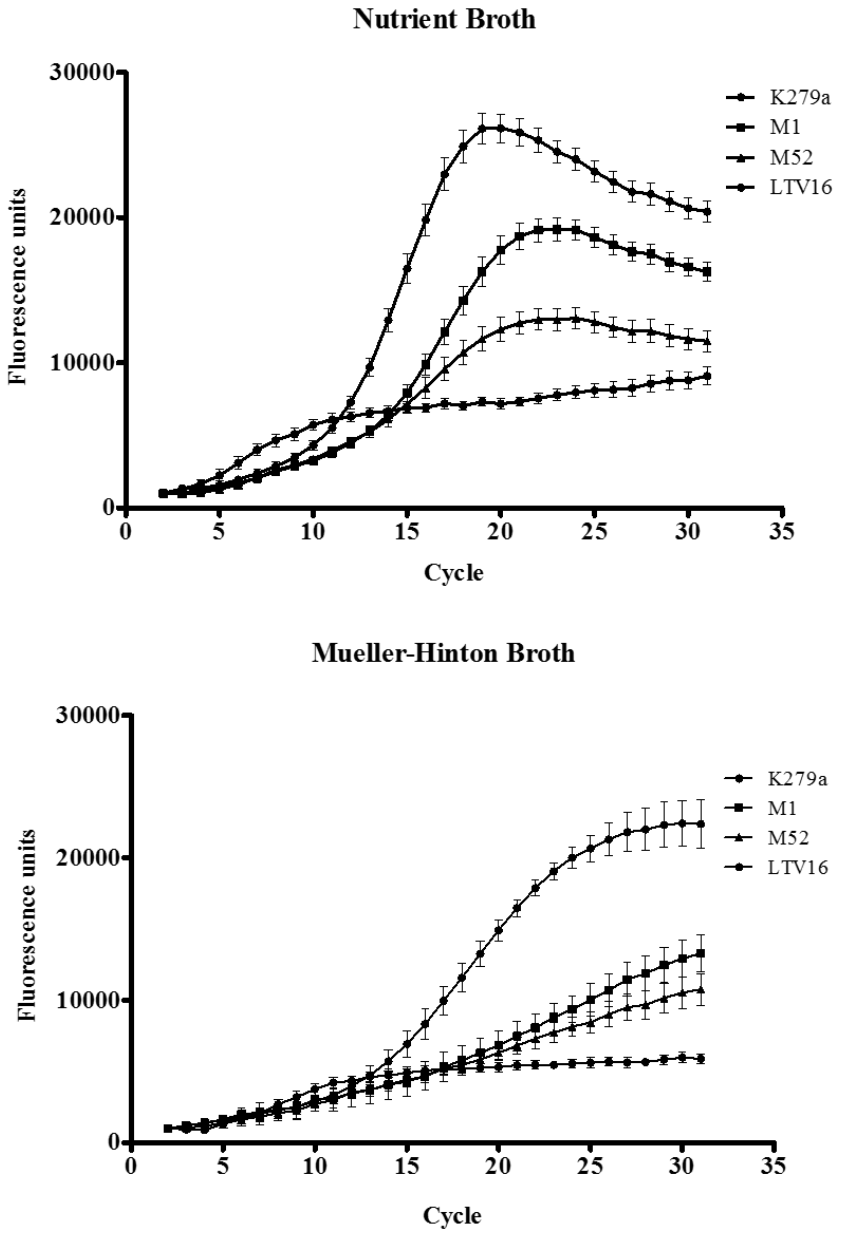


Figure 6.4 Envelope permeability measured by absolute accumulation of H33342 in low-osmolality (NB) and high-osmolality (MHB) media

Absolute accumulation of H33342 (25 µM) is expressed as fluorescence units over 31 cycle incubation period (78 min). Each curve plots data for three biological replicates with eight technical replicates. Error bars represent standard estimation of the mean (SEM)

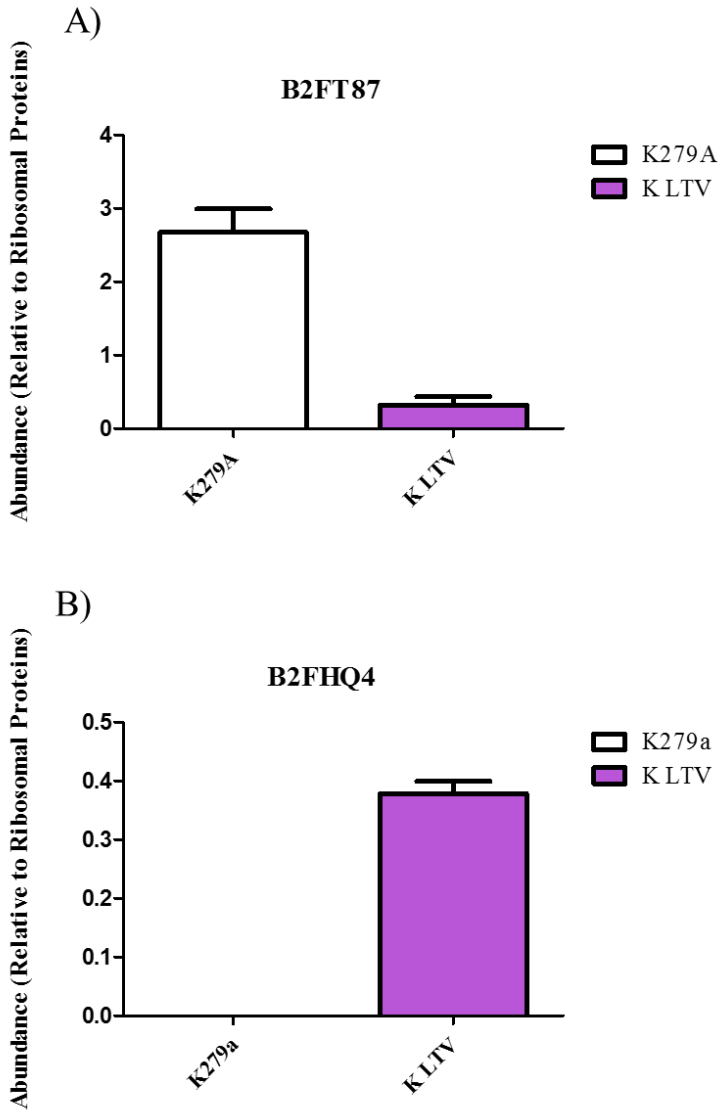


Figure 6.5 A) Downregulation of the TonB energy transducer protein Smlt0009 and B) upregulation of EntB siderophore biosynthesis enzyme in K LTV

Protein abundance data of whole envelope proteomics are normalised to the average ribosomal content of each sample (including abundance of 30S and 50S ribosomal proteins). **A)** Fold change of Smlt0009 (Uniprot: B2FT87) and **B)** EntB (Uniprot: B2FH84) is calculated after testing statistical significant difference between the parental strain and the mutants (p <0.05). Values are reported as mean +/- Standard Error of the Mean (n=3). Full Proteomics data are shown in **Annex**

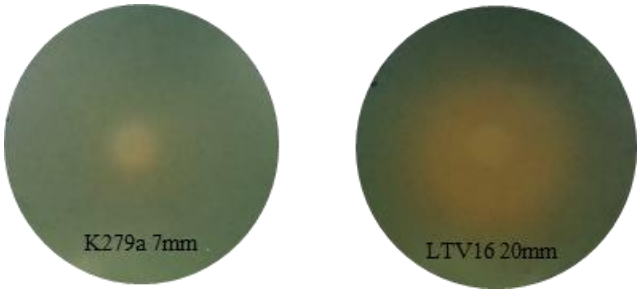


Figure 6.6 Siderophore overproduction of K LTV

Diameter values show diffusion of the siderophore production after spotting 10 µL of a PBS washed bacterial suspension (OD_{600} 0.2) onto a modified CAS agar (as described in section **2.4**). Values are reported as mean of three biological repeats; the figure is representative

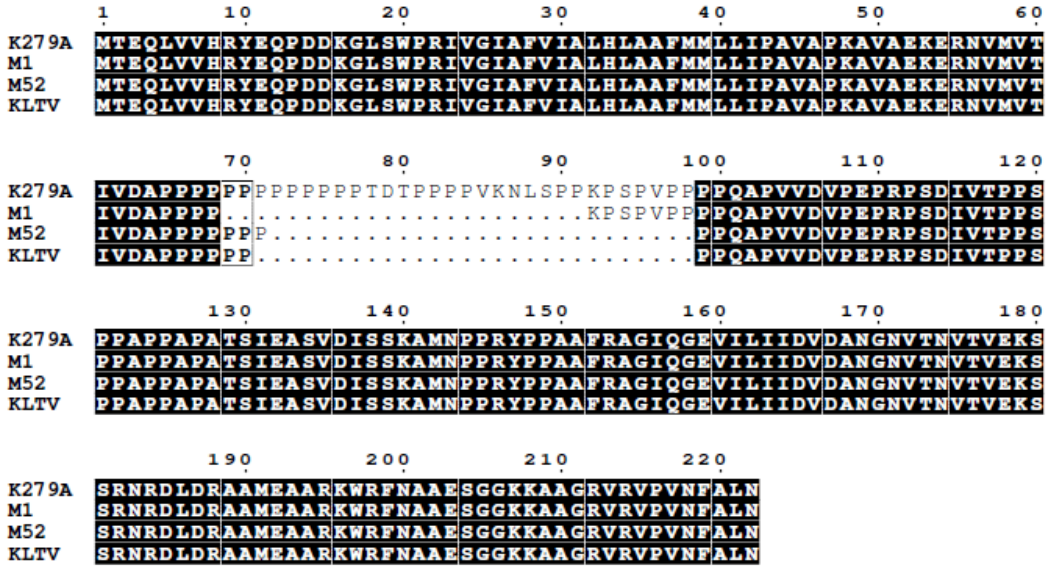


Figure 6.7 Sequence alignment of energy transducer TonB of ceftazidime and lactivicin resistant mutants

Alignment of translated PCR sequences that confirmed loss of proline-rich region (non-highlighted region) in M1 and M52 mutants. PCR amplification used a found during whole genome sequencing analysis. Alignment was performed with CLUSTAL Omega and ESPript 3.x

6.3 Conclusions

The side chain modification of the core fused bicyclic non β-lactam ring system of the Lactivicins has the potential to improve activity in the same way as it has done for the β-lactams, e.g.BAL30072, which is in early phase clinical development. This can work by increasing potency versus PBPs and/or reducing β-lactamase susceptibility, and in addition by improving uptake (354). Moreover, the results here presented suggest that siderophore mediated uptake is not a general effect, equally seen in all species. It may be that the apparent species-specificity of the effect seen in dependent on the conjugation of a particular siderophore preferentially used by a particular species. In an era of improved diagnostics for infection (355), the routine use of narrow-spectrum antimicrobials becomes a realistic proposition, and the benefit would be reduced collateral damage to the host microbiome, and cross-selection for the acquisition of resistant isolates of other species of bacteria. Within the time allowed, we have found enough evidence to suggest the participation of the TonB energy transducer is not only important for ceftazidime uptake but also uptake of lactivicins even when they have been modified with a sideromimic group which fits well with what has been seen before for mutants resistant to siderophore-conjugated antimicrobials. Whilst the MIC of LTV17 is 0.5mg.L^{-1} against these mutants, we do not know whether this would be classed as clinically resistant, because LTV17 is an experimental drug and has not yet been administered in humans. Accordingly, it is still possible that the mutation does not confer clinical resistance.

6.4 Annex 4

Table 6.5 Normalised proteomics data for K LTV against K279a

Abundance changes significantly according to a t-Test (P<0.05). Proteins that are >1.5 fold up and down regulated are highlighted green or red, respectively.

Accession	Description	T-Test $_{K279a\ vs\ K\ LTV}$	Fold $_{K279a\ vs\ K\ LTV}$
B2FHA2	Putative ferredoxin oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0136 PE=4 SV=1 - [B2FHA2_STRMK]	0.039	0.13
B2FHA8	Putative phospholipase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0142 PE=4 SV=1 - [B2FHA8_STRMK]	0.007	0.43
B2FHB5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0149 PE=4 SV=1 - [B2FHB5_STRMK]	0.004	0.38
B2FHB7	Glutamine synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=glnA PE=3 SV=1 - [B2FHB7_STRMK]	<0.05	>100
B2FHC6	Superoxide dismutase [Cu-Zn] OS=Stenotrophomonas maltophilia (strain K279a) GN=sodC1 PE=3 SV=1 - [B2FHC6_STRMK]	0.003	0.38
B2FHC7	Superoxide dismutase [Cu-Zn] OS=Stenotrophomonas maltophilia (strain K279a) GN=sodC2 PE=3 SV=1 - [B2FHC7_STRMK]	0.032	0.49
B2FHD0	Putative 3-ketoacyl-CoA thiolase OS=Stenotrophomonas maltophilia (strain K279a) GN=fadI PE=3 SV=1 - [B2FHD0_STRMK]	0.016	0.48
B2FHD6	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0170 PE=4 SV=1 - [B2FHD6_STRMK]	0.007	0.41
B2FHE8	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0182 PE=4 SV=1 - [B2FHE8_STRMK]	<0.05	2.85
B2FHF0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0184 PE=4 SV=1 - [B2FHF0_STRMK]	0.006	0.39
B2FHF9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0193 PE=4 SV=1 - [B2FHF9_STRMK]	<0.05	>100
B2FHG4	Putative electron transfer flavoprotein-ubiquinone oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0198 PE=4 SV=1 - [B2FHG4_STRMK]	0.004	0.28
B2FHH2	Putative TonB dependent siderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1426 PE=3 SV=1 - [B2FHH2_STRMK]	0.000	18.96
B2FHH7	Putative peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1431 PE=4 SV=1 - [B2FHH7_STRMK]	0.012	0.36
B2FHI7	Glutamate--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=gltX PE=3 SV=1 - [SYE_STRMK]	<0.05	0.41
B2FHI8	Putative regulatory protein OS=Stenotrophomonas maltophilia (strain K279a) GN=fur PE=4 SV=1 - [B2FHI8_STRMK]	<0.05	>100
B2FHJ2	Putative outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1446 PE=3 SV=1 - [B2FHJ2_STRMK]	0.001	0.17
B2FHJ5	Putative phosphodiesterase-nucleotide pyrophosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1449 PE=4 SV=1 - [B2FHJ5_STRMK]	0.003	0.31
B2FHK0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1454 PE=4 SV=1 - [B2FHK0_STRMK]	<0.05	0.19
B2FHL6	Putative ABC transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1471 PE=3 SV=1 - [B2FHL6_STRMK]	0.019	0.38
B2FHL9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1474 PE=4 SV=1 - [B2FHL9_STRMK]	0.009	0.41
B2FHN2	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1491 PE=4 SV=1 - [B2FHN2_STRMK]	0.017	0.43
B2FHN5	Lipid-A-disaccharide synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=lpxB PE=3 SV=1 - [B2FHN5_STRMK]	0.013	0.48
B2FHQ1	Putative enterobactin synthetase component A (2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase) OS=Stenotrophomonas maltophilia (strain	<0.05	>100

	K279a) GN=entA PE=4 SV=1 - [B2FHQ1_STRMK]		
B2FHQ4	**Putative hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2820 PE=4 SV=1 - [B2FHQ4_STRMK]**	**<0.05**	**>100**
B2FHQ5	Putative siderophore specific 2,3-dihydroxybenzoate-AMP ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2821 PE=4 SV=1 - [B2FHQ5_STRMK]	<0.05	>100
B2FHR5	Putative transmembrane LINOLEOYL-CoA DESATURASE (DELTA(6)-DESATURASE) OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2831 PE=4 SV=1 - [B2FHR5_STRMK]	<0.05	0.42
B2FHR8	Putative autotransporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2834 PE=4 SV=1 - [B2FHR8_STRMK]	0.032	0.26
B2FHR9	Putative TonB-dependent outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2835 PE=3 SV=1 - [B2FHR9_STRMK]	0.000	3.57
B2FHT4	Putative TonB dependent extracellular heme-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2850 PE=3 SV=1 - [B2FHT4_STRMK]	<0.05	>100
B2FHT9	Putative iron transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2858 PE=3 SV=1 - [B2FHT9_STRMK]	0.000	4.19
B2FHW5	Methionine import ATP-binding protein MetN OS=Stenotrophomonas maltophilia (strain K279a) GN=metN PE=3 SV=1 - [B2FHW5_STRMK]	0.012	0.34
B2FHX5	Putative CBS domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4098 PE=4 SV=1 - [B2FHX5_STRMK]	<0.05	>100
B2FHY7	ATP synthase epsilon chain OS=Stenotrophomonas maltophilia (strain K279a) GN=atpC PE=3 SV=1 - [ATPE_STRMK]	0.002	0.43
B2FHZ2	ATP synthase subunit b OS=Stenotrophomonas maltophilia (strain K279a) GN=atpF PE=3 SV=1 - [ATPF_STRMK]	0.013	0.40
B2FHZ7	Putative dihydrolipoamide dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=lpdA PE=4 SV=1 - [B2FHZ7_STRMK]	0.001	2.48
B2FHZ8	Acetyltransferase component of pyruvate dehydrogenase complex OS=Stenotrophomonas maltophilia (strain K279a) GN=pdhB PE=3 SV=1 - [B2FHZ8_STRMK]	0.012	2.71
B2FI00	Putative outer membrane Omp family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4123 PE=4 SV=1 - [B2FI00_STRMK]	0.002	0.25
B2FI05	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4128 PE=4 SV=1 - [B2FI05_STRMK]	0.006	0.45
B2FI10	Glucans biosynthesis glucosyltransferase H OS=Stenotrophomonas maltophilia (strain K279a) GN=opgH PE=3 SV=1 - [B2FI10_STRMK]	<0.05	>100
B2FI12	Putative colicin I receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=cirA PE=3 SV=1 - [B2FI12_STRMK]	0.000	75.12
B2FI15	Putative methyl-accepting chemotaxis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4138 PE=4 SV=1 - [B2FI15_STRMK]	<0.05	>100
B2FI22	Putative extracellular serine protease OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4145 PE=4 SV=1 - [B2FI22_STRMK]	<0.05	>100
B2FI29	Putative NAD(P)H dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0209 PE=4 SV=1 - [B2FI29_STRMK]	0.011	0.19
B2FI43	Putative peptidyl-dipeptidase Dcp (Dipeptidyl carboxypeptidase) OS=Stenotrophomonas maltophilia (strain K279a) GN=dcp PE=3 SV=1 - [B2FI43_STRMK]	0.010	0.41
B2FI64	Ribonucleoside-diphosphate reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=RRM1 PE=3 SV=1 - [B2FI64_STRMK]	0.008	3.67
B2FI80	DntE OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0264 PE=3 SV=1 - [B2FI80_STRMK]	<0.05	0.39
B2FIA0	Outer membrane protein assembly factor BamA OS=Stenotrophomonas maltophilia (strain K279a) GN=bamA PE=3 SV=1 - [B2FIA0_STRMK]	0.010	0.48
B2FIA8	Elongation factor Ts OS=Stenotrophomonas maltophilia (strain K279a) GN=tsf PE=3 SV=1 - [EFTS_STRMK]	0.015	2.07
B2FIC9	Putative multidrug resistance protein A OS=Stenotrophomonas maltophilia (strain K279a) GN=emrA PE=4 SV=1 - [B2FIC9_STRMK]	0.025	0.37
B2FIE0	Putative two-component system, response regulator transcriptional regulatory protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1540 PE=4 SV=1 - [B2FIE0_STRMK]	<0.05	0.47
B2FII5	Putative CDP-diacylglycerol pyrophosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2903 PE=4 SV=1 - [B2FII5_STRMK]	0.009	0.37
B2FIL8	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2937 PE=3 SV=1 - [B2FIL8_STRMK]	<0.05	>100
B2FIM0	Putative TonB-like protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2939 PE=4 SV=1 - [B2FIM0_STRMK]	<0.05	>100
B2FIQ4	GTP cyclohydrolase II OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4168 PE=3 SV=1 - [B2FIQ4_STRMK]	<0.05	0.42
B2FIR5	Putative LysM family cell wall degradation protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4179 PE=4 SV=1 - [B2FIR5_STRMK]	0.035	0.12

B2FIR9	Putative transmembrane RDD family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4183 PE=4 SV=1 - [B2FIR9_STRMK]	0.016	0.47
B2FIS0	DNA topoisomerase 1 OS=Stenotrophomonas maltophilia (strain K279a) GN=topA PE=3 SV=1 - [B2FIS0_STRMK]	<0.05	>100
B2FIS4	Putative lipid biosynthesis 3-oxoacyl-[acyl-carrier-protein] reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4188 PE=4 SV=1 - [B2FIS4_STRMK]	<0.05	0.46
B2FIS6	Protease 4 OS=Stenotrophomonas maltophilia (strain K279a) GN=sppA PE=3 SV=1 - [B2FIS6_STRMK]	0.002	0.38
B2FIS7	Putative drug resistance transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4191 PE=4 SV=1 - [B2FIS7_STRMK]	0.005	0.43
B2FIU9	60 kDa chaperonin OS=Stenotrophomonas maltophilia (strain K279a) GN=groL PE=3 SV=1 - [CH60_STRMK]	0.001	2.22
B2FIV0	10 kDa chaperonin OS=Stenotrophomonas maltophilia (strain K279a) GN=groS PE=3 SV=1 - [CH10_STRMK]	0.000	3.64
B2FJ30	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0359 PE=3 SV=1 - [B2FJ30_STRMK]	0.022	0.33
B2FJ60	Putative transmembrane PepSY domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1566 PE=4 SV=1 - [B2FJ60_STRMK]	<0.05	>100
B2FJ77	Polyamine-transporting ATPase OS=Stenotrophomonas maltophilia (strain K279a) GN=potG PE=3 SV=1 - [B2FJ77_STRMK]	0.013	0.36
B2FJ78	Putative putrescine transport system permease protein OS=Stenotrophomonas maltophilia (strain K279a) GN=potH PE=3 SV=1 - [B2FJ78_STRMK]	0.007	0.32
B2FJ81	Putative transmembrane magnesium/cobalt transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=corA PE=4 SV=1 - [B2FJ81_STRMK]	0.039	0.49
B2FJB1	Putative oar family adhesion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1619 PE=4 SV=1 - [B2FJB1_STRMK]	0.002	0.32
B2FJB8	Putative PilE protein (Type 4 fimbrial biogenesis protein PilE) OS=Stenotrophomonas maltophilia (strain K279a) GN=pilE PE=4 SV=1 - [B2FJB8_STRMK]	0.033	0.43
B2FJC0	UvrABC system protein B OS=Stenotrophomonas maltophilia (strain K279a) GN=uvrB PE=3 SV=1 - [B2FJC0_STRMK]	<0.05	0.43
B2FJC8	Histidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1636 PE=4 SV=1 - [B2FJC8_STRMK]	0.030	0.47
B2FJG0	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2989 PE=4 SV=1 - [B2FJG0_STRMK]	0.027	0.42
B2FJJ3	Putative hydroxamate-type ferrisiderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3022 PE=3 SV=1 - [B2FJJ3_STRMK]	0.000	22.50
B2FJP1	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4245 PE=4 SV=1 - [B2FJP1_STRMK]	0.017	0.44
B2FJQ7	Putative cell division ATP-binding protein FtsE OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsE PE=3 SV=1 - [B2FJQ7_STRMK]	0.013	0.39
B2FJR1	Putative transmembrane GGDEF signalling protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4266 PE=4 SV=1 - [B2FJR1_STRMK]	0.021	0.48
B2FJR8	Putative isocitrate dehydrogenase [NADP] OS=Stenotrophomonas maltophilia (strain K279a) GN=icd PE=4 SV=1 - [B2FJR8_STRMK]	0.002	4.57
B2FJS3	Putative multidrug efflux system HlyD family transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4279 PE=4 SV=1 - [B2FJS3_STRMK]	0.007	0.36
B2FJS5	Putative AcrB/AcrD/AcrF family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4281 PE=4 SV=1 - [B2FJS5_STRMK]	<0.05	>100
B2FJV0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0387 PE=4 SV=1 - [B2FJV0_STRMK]	0.002	0.39
B2FJV8	Putative oxidoreductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0396 PE=3 SV=1 - [B2FJV8_STRMK]	<0.05	0.49
B2FJV9	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0397 PE=4 SV=1 - [B2FJV9_STRMK]	0.020	0.48
B2FJW5	Putative phosphomannomutase OS=Stenotrophomonas maltophilia (strain K279a) GN=spgM PE=4 SV=1 - [B2FJW5_STRMK]	0.022	0.42
B2FK31	Putative ABC transporter ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1653 PE=3 SV=1 - [B2FK31_STRMK]	<0.05	>100
B2FK68	Putative glutamate synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1693 PE=3 SV=1 - [B2FK68_STRMK]	0.003	0.22
B2FK78	Putative pit accessory protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1705 PE=4 SV=1 - [B2FK78_STRMK]	0.020	0.46
B2FK79	Putative LOW-AFFINITY INORGANIC PHOSPHATE TRANSPORTER INTEGRAL MEMBRANE PROTEIN PITA OS=Stenotrophomonas maltophilia (strain K279a) GN=pitA PE=4 SV=1 - [B2FK79_STRMK]	0.022	0.41
B2FK80	Putative carboxypeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1707 PE=4 SV=1 - [B2FK80_STRMK]	<0.05	>100

B2FK88	Enolase OS=Stenotrophomonas maltophilia (strain K279a) GN=eno PE=3 SV=1 - [ENO_STRMK]	<0.05	12.72
B2FK97	Putative subfamily M23B unassigned peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1724 PE=4 SV=1 - [B2FK97_STRMK]	0.003	0.36
B2FKE9	Putative P-protein [bifunctional includes: chorismate mutase and prephenate dehydratase OS=Stenotrophomonas maltophilia (strain K279a) GN=pheA PE=4 SV=1 - [B2FKE9_STRMK]	0.012	0.08
B2FKF1	Conserved hypothetical FHA domain protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3099 PE=4 SV=1 - [B2FKF1_STRMK]	0.021	0.49
B2FKG5	Putative TonB-dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3115 PE=4 SV=1 - [B2FKG5_STRMK]	0.010	0.47
B2FKH5	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3125 PE=4 SV=1 - [B2FKH5_STRMK]	<0.05	0.35
B2FKJ7	50S ribosomal protein L9 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplI PE=3 SV=1 - [RL9_STRMK]	0.003	2.68
B2FKJ9	30S ribosomal protein S6 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsF PE=3 SV=1 - [B2FKJ9_STRMK]	0.003	2.62
B2FKK8	Putative cyclic AMP receptor protein,catabolite gene activator OS=Stenotrophomonas maltophilia (strain K279a) GN=cap PE=4 SV=1 - [B2FKK8_STRMK]	0.001	2.43
B2FKM2	Putative K(+)/H(+) antiporter subunit A/B (PH adaptation potassium efflux system protein A/B) OS=Stenotrophomonas maltophilia (strain K279a) GN=phaAB PE=4 SV=1 - [B2FKM2_STRMK]	<0.05	0.35
B2FKR5	Putative nitrogen regulatory protein P-II OS=Stenotrophomonas maltophilia (strain K279a) GN=glnB PE=3 SV=1 - [B2FKR5_STRMK]	<0.05	>100
B2FKV5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0481 PE=4 SV=1 - [B2FKV5_STRMK]	<0.05	0.47
B2FKV6	Putative NADH dehydrogenase/NAD(P)H nitroreductase Smlt0482 OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0482 PE=3 SV=1 - [Y482_STRMK]	<0.05	2.24
B2FKW2	Pyruvate dehydrogenase E1 component OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0490 PE=4 SV=1 - [B2FKW2_STRMK]	0.010	3.25
B2FKX4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0503 PE=4 SV=1 - [B2FKX4_STRMK]	<0.05	>100
B2FL00	Putative NHL repeat protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0528 PE=4 SV=1 - [B2FL00_STRMK]	0.027	0.42
B2FL08	Putative transmembrane anchor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0538 PE=4 SV=1 - [B2FL08_STRMK]	0.002	0.22
B2FL10	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0540 PE=4 SV=1 - [B2FL10_STRMK]	0.002	0.26
B2FL11	Putative aminopeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0541 PE=4 SV=1 - [B2FL11_STRMK]	<0.05	0.23
B2FL46	Putative cytochrome c family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1757 PE=4 SV=1 - [B2FL46_STRMK]	<0.05	>100
B2FL47	Putative alcohol dehydrogenase cytochrome c subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=adhB PE=4 SV=1 - [B2FL47_STRMK]	<0.05	>100
B2FL51	Putative ferric siderophore receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1762 PE=3 SV=1 - [B2FL51_STRMK]	<0.05	>100
B2FL84	Putative succinate dehydrogenase cytochrome b-556 subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=sdhC PE=4 SV=1 - [B2FL84_STRMK]	0.002	0.26
B2FL86	Putative succinate dehydrogenase flavoprotein subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=sdhA PE=4 SV=1 - [B2FL86_STRMK]	0.006	0.20
B2FL87	Succinate dehydrogenase iron-sulfur subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=sdhB PE=3 SV=1 - [B2FL87_STRMK]	0.002	0.23
B2FLA6	Putative RND family acriflavine resistance protein A OS=Stenotrophomonas maltophilia (strain K279a) GN=smeG PE=4 SV=1 - [B2FLA6_STRMK]	0.011	0.48
B2FLA7	Putative multidrug resistance efflux pump OS=Stenotrophomonas maltophilia (strain K279a) GN=smeH PE=4 SV=1 - [B2FLA7_STRMK]	0.020	0.41
B2FLB8	Peptidyl-prolyl cis-trans isomerase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3182 PE=4 SV=1 - [B2FLB8_STRMK]	0.005	0.35
B2FLC5	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3189 PE=4 SV=1 - [B2FLC5_STRMK]	<0.05	2.04
B2FLD1	Putative 2-oxoglutarate dehydrogenase E1 component OS=Stenotrophomonas maltophilia (strain K279a) GN=sucA PE=4 SV=1 - [B2FLD1_STRMK]	0.027	2.60
B2FLE4	Putative outer membrane antigen protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3210 PE=4 SV=1 - [B2FLE4_STRMK]	0.036	0.49
B2FLE9	Putative outer membrane antigen lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3215 PE=4 SV=1 - [B2FLE9_STRMK]	0.015	0.45
B2FLF2	Putative phospholipase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3218 PE=4 SV=1 - [B2FLF2_STRMK]	0.005	0.41
B2FLG5	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3232 PE=4 SV=1 - [B2FLG5_STRMK]	0.006	0.40

B2FLG9	Putative peptide transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3236 PE=3 SV=1 - [B2FLG9_STRMK]	0.029	0.48
B2FLH1	Superoxide dismutase OS=Stenotrophomonas maltophilia (strain K279a) GN=sodA PE=3 SV=1 - [B2FLH1_STRMK]	0.000	2.77
B2FLH4	Putative transmembrane repetitive protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3241 PE=4 SV=1 - [B2FLH4_STRMK]	0.027	4.81
B2FLP0	Putative transmembrane Na+/H+ antiport transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0555 PE=4 SV=1 - [B2FLP0_STRMK]	0.038	0.49
B2FLQ1	D-amino acid dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=dadA PE=3 SV=1 - [DADA_STRMK]	0.016	0.32
B2FLR8	Putative vitamin B12 receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0585 PE=3 SV=1 - [B2FLR8_STRMK]	0.006	0.48
B2FLT1	Asparagine synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0598 PE=4 SV=1 - [B2FLT1_STRMK]	<0.05	>100
B2FLU8	Transport permease protein OS=Stenotrophomonas maltophilia (strain K279a) GN=wzm PE=3 SV=1 - [B2FLU8_STRMK]	0.021	0.49
B2FLV4	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0624 PE=4 SV=1 - [B2FLV4_STRMK]	0.041	0.49
B2FLV6	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0626 PE=4 SV=1 - [B2FLV6_STRMK]	0.010	0.34
B2FLW2	Chaperone protein HtpG OS=Stenotrophomonas maltophilia (strain K279a) GN=htpG PE=3 SV=1 - [B2FLW2_STRMK]	0.007	4.25
B2FLW5	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1812 PE=4 SV=1 - [B2FLW5_STRMK]	0.015	0.46
B2FLX9	Putative outer membrane lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1826 PE=3 SV=1 - [B2FLX9_STRMK]	0.011	0.43
B2FLZ4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1841 PE=4 SV=1 - [B2FLZ4_STRMK]	<0.05	0.42
B2FM92	Ribonuclease E OS=Stenotrophomonas maltophilia (strain K279a) GN=rnE PE=3 SV=1 - [B2FM92_STRMK]	0.023	2.36
B2FMB8	Putative cytochrome C-type biogenesis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=dsbE PE=4 SV=1 - [B2FMB8_STRMK]	0.027	0.26
B2FMC4	Putative ABC transporter ATP-binding protein, cytochrome related OS=Stenotrophomonas maltophilia (strain K279a) GN=cydD PE=3 SV=1 - [B2FMC4_STRMK]	<0.05	0.25
B2FMF2	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3312 PE=4 SV=1 - [B2FMF2_STRMK]	0.015	0.46
B2FMI1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4498 PE=4 SV=1 - [B2FMI1_STRMK]	0.009	0.45
B2FMI9	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4506 PE=4 SV=1 - [B2FMI9_STRMK]	0.010	0.40
B2FMK1	Putative ABC transporter ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4518 PE=3 SV=1 - [B2FMK1_STRMK]	0.014	0.46
B2FML3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4532 PE=4 SV=1 - [B2FML3_STRMK]	<0.05	>100
B2FMN5	Putative transmembrane UbiA prenyltransferase family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0634 PE=4 SV=1 - [B2FMN5_STRMK]	0.012	0.37
B2FMP6	Putative electron transfer flavoprotein subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=etfS PE=4 SV=1 - [B2FMP6_STRMK]	0.001	3.18
B2FMP8	Glucose-1-phosphate thymidylyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=rmlA PE=3 SV=1 - [B2FMP8_STRMK]	<0.05	>100
B2FMQ1	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0651 PE=4 SV=1 - [B2FMQ1_STRMK]	0.021	0.39
B2FMR2	Putative permease component of ABC transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0662 PE=4 SV=1 - [B2FMR2_STRMK]	0.000	0.28
B2FMR3	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0663 PE=4 SV=1 - [B2FMR3_STRMK]	0.002	2.24
B2FMR8	Putative transmembrane CDP-diacylglycerol--serine O-phosphatidyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0669 PE=3 SV=1 - [B2FMR8_STRMK]	0.023	0.44
B2FMS5	Putative transmembrane permease protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0676 PE=4 SV=1 - [B2FMS5_STRMK]	0.008	0.39
B2FMS6	Putative transmembrane permease protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0677 PE=4 SV=1 - [B2FMS6_STRMK]	0.026	0.45
B2FMX9	Putative ferric uptake regulation protein OS=Stenotrophomonas maltophilia (strain K279a) GN=fur PE=4 SV=1 - [B2FMX9_STRMK]	<0.05	2.61
B2FN02	Protein translocase subunit SecD OS=Stenotrophomonas maltophilia (strain K279a) GN=secD PE=3 SV=1 - [B2FN02_STRMK]	0.016	0.41
B2FN03	Protein-export membrane protein SecF OS=Stenotrophomonas maltophilia (strain K279a) GN=secF PE=3 SV=1 - [B2FN03_STRMK]	0.016	0.47

B2FN25	Putative carbon-nitrogen hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2038 PE=4 SV=1 - [B2FN25_STRMK]	<0.05	>100
B2FN37	Putative N-acetylmuramoyl-L-alanine amidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3330 PE=4 SV=1 - [B2FN37_STRMK]	0.006	0.29
B2FN47	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3340 PE=3 SV=1 - [B2FN47_STRMK]	0.003	0.27
B2FN55	Putative acyl-CoA dehydrogenase oxidoreductase protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3352 PE=3 SV=1 - [B2FN55_STRMK]	<0.05	>100
B2FN59	Conserved hypothetical repetitive protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3358 PE=4 SV=1 - [B2FN59_STRMK]	0.006	0.34
B2FN90	Translation initiation factor IF-2 OS=Stenotrophomonas maltophilia (strain K279a) GN=infB PE=3 SV=1 - [IF2_STRMK]	0.042	2.48
B2FN93	NADH-quinone oxidoreductase subunit N OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoN PE=3 SV=1 - [B2FN93_STRMK]	0.022	0.27
B2FN94	Putative NADH dehydrogenase I chain M OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoM PE=4 SV=1 - [B2FN94_STRMK]	0.016	0.21
B2FN95	Putative NADH-ubiquinone oxidoreductase I chain L OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoL PE=4 SV=1 - [B2FN95_STRMK]	0.020	0.25
B2FNA4	Putative exported lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4560 PE=4 SV=1 - [B2FNA4_STRMK]	0.029	0.21
B2FNA6	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4562 PE=4 SV=1 - [B2FNA6_STRMK]	0.021	0.48
B2FNC5	Putative dipeptidyl peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4581 PE=4 SV=1 - [B2FNC5_STRMK]	0.004	0.42
B2FND7	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4593 PE=4 SV=1 - [B2FND7_STRMK]	0.002	0.30
B2FNF1	Putative transmembrane efflux pump protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smmQ PE=4 SV=1 - [B2FNF1_STRMK]	<0.05	0.38
B2FNF4	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4611 PE=4 SV=1 - [B2FNF4_STRMK]	0.003	0.26
B2FNG6	Acetyl-coenzyme A synthetase OS=Stenotrophomonas maltophilia (strain K279a) GN=acsA PE=3 SV=1 - [B2FNG6_STRMK]	<0.05	>100
B2FNK2	Serine hydroxymethyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=glyA PE=3 SV=1 - [GLYA_STRMK]	<0.05	>100
B2FNL0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0726 PE=4 SV=1 - [B2FNL0_STRMK]	0.045	0.44
B2FNN2	Putative penicillin-binding protein 3 OS=Stenotrophomonas maltophilia (strain K279a) GN=ftsI PE=4 SV=1 - [B2FNN2_STRMK]	0.005	0.37
B2FNN3	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=murE PE=3 SV=1 - [B2FNN3_STRMK]	<0.05	0.27
B2FNN7	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=murG PE=3 SV=1 - [B2FNN7_STRMK]	0.012	0.45
B2FNP5	30S ribosomal protein S1 OS=Stenotrophomonas maltophilia (strain K279a) GN=rpsA PE=3 SV=1 - [B2FNP5_STRMK]	0.001	3.19
B2FNQ8	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2057 PE=4 SV=1 - [B2FNQ8_STRMK]	0.045	0.46
B2FNQ9	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2058 PE=4 SV=1 - [B2FNQ9_STRMK]	0.020	0.36
B2FNS0	Inosine-5'-monophosphate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=guaB PE=3 SV=1 - [B2FNS0_STRMK]	0.005	5.15
B2FNX0	Putative NADH-ubiquinone oxidoreductase, chain J OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoJ PE=3 SV=1 - [B2FNX0_STRMK]	<0.05	0.15
B2FNX1	NADH-quinone oxidoreductase subunit I OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoI PE=3 SV=1 - [B2FNX1_STRMK]	0.004	0.23
B2FNX2	NADH-quinone oxidoreductase subunit H OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoH PE=3 SV=1 - [B2FNX2_STRMK]	0.005	0.11
B2FNX3	Putative NADH-ubiquinone oxidoreductase, 75 kDa subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoG PE=4 SV=1 - [B2FNX3_STRMK]	0.009	0.19
B2FNX4	Putative NADH dehydrogenase I chain F OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoF PE=4 SV=1 - [B2FNX4_STRMK]	0.019	0.19
B2FNX5	Putative respiratory-chain NADH dehydrogenase I, 24 kDa subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoE PE=4 SV=1 - [B2FNX5_STRMK]	<0.05	>100
B2FNX6	NADH-quinone oxidoreductase subunit D OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoD PE=3 SV=1 - [NUOD_STRMK]	0.003	0.32
B2FNX7	NADH-quinone oxidoreductase subunit C OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoC PE=3 SV=1 - [NUOC_STRMK]	0.001	0.21
B2FNX8	NADH-quinone oxidoreductase subunit B OS=Stenotrophomonas maltophilia (strain K279a) GN=nuoB PE=3 SV=1 - [NUOB_STRMK]	0.002	0.23

B2FNY0	Putative general secretory pathway protein-export membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=secG PE=4 SV=1 - [B2FNY0_STRMK]	0.002	0.42
B2FNY5	Putative lipopolysaccharide core oligosaccharide biosynthesis protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3412 PE=4 SV=1 - [B2FNY5_STRMK]	0.010	0.31
B2FNZ9	Aspartate-semialdehyde dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=asd PE=3 SV=1 - [B2FNZ9_STRMK]	<0.05	0.44
B2FP18	Putative endopeptidase O OS=Stenotrophomonas maltophilia (strain K279a) GN=pepO PE=4 SV=1 - [B2FP18_STRMK]	0.019	0.48
B2FP46	Putative surface antigen exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4633 PE=4 SV=1 - [B2FP46_STRMK]	0.021	0.49
B2FP53	Sec-independent protein translocase protein TatB OS=Stenotrophomonas maltophilia (strain K279a) GN=tatB PE=3 SV=1 - [B2FP53_STRMK]	0.008	0.40
B2FP71	GTP cyclohydrolase 1 OS=Stenotrophomonas maltophilia (strain K279a) GN=folE PE=3 SV=1 - [B2FP71_STRMK]	<0.05	>100
B2FP87	Putative intercellular spreading VacJ lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4675 PE=4 SV=1 - [B2FP87_STRMK]	0.011	0.47
B2FP99	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4687 PE=4 SV=1 - [B2FP99_STRMK]	<0.05	>100
B2FPA4	Putative polysaccharide deacetylase family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4692 PE=4 SV=1 - [B2FPA4_STRMK]	0.012	0.41
B2FPA5	Membrane protein insertase YidC OS=Stenotrophomonas maltophilia (strain K279a) GN=yidC PE=3 SV=1 - [YIDC_STRMK]	0.010	0.46
B2FPC4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0777 PE=4 SV=1 - [B2FPC4_STRMK]	<0.05	0.19
B2FPD1	Putative glycosyl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0784 PE=4 SV=1 - [B2FPD1_STRMK]	0.019	0.47
B2FPD9	Probable Na+ dependent nucleoside transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0792 PE=4 SV=1 - [B2FPD9_STRMK]	0.001	0.44
B2FPE2	Putative exported heme receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=huvA PE=3 SV=1 - [B2FPE2_STRMK]	0.000	42.38
B2FPE3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0796 PE=4 SV=1 - [B2FPE3_STRMK]	<0.05	>100
B2FPE4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0797 PE=4 SV=1 - [B2FPE4_STRMK]	<0.05	>100
B2FPF2	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0805 PE=4 SV=1 - [B2FPF2_STRMK]	0.006	0.39
B2FPF9	Prolipoprotein diacylglyceryl transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=lgt PE=3 SV=1 - [LGT_STRMK]	0.010	0.43
B2FPH9	Glutamine--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=glnS PE=3 SV=1 - [B2FPH9_STRMK]	<0.05	0.08
B2FPK0	Putative repetitive protein with two-component sensor and regulator motifs OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2141 PE=4 SV=1 - [B2FPK0_STRMK]	0.012	0.24
B2FPM6	Putative calcineurin phosphoesterase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2170 PE=4 SV=1 - [B2FPM6_STRMK]	<0.05	0.48
B2FPN5	Putative TonB-dependent outer membrane receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2179 PE=3 SV=1 - [B2FPN5_STRMK]	0.006	0.23
B2FPP8	Putative amino-acid transporter transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2193 PE=4 SV=1 - [B2FPP8_STRMK]	0.002	0.40
B2FPQ0	Putative D-3-phosphoglycerate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=serA PE=3 SV=1 - [B2FPQ0_STRMK]	<0.05	>100
B2FPR6	LPS-assembly lipoprotein LptE OS=Stenotrophomonas maltophilia (strain K279a) GN=lptE PE=3 SV=1 - [B2FPR6_STRMK]	0.008	0.46
B2FPS0	Putative exported LysM bacterial cell wall related protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3488 PE=4 SV=1 - [B2FPS0_STRMK]	0.004	0.28
B2FPS1	Putative transmembrane GGDEF transcriptional regulatory protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3489 PE=4 SV=1 - [B2FPS1_STRMK]	<0.05	>100
B2FPV6	Putative thiolase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3525 PE=3 SV=1 - [B2FPV6_STRMK]	<0.05	0.37
B2FPY0	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3550 PE=4 SV=1 - [B2FPY0_STRMK]	0.007	0.38
B2FPY7	Putative alkyl hydroperoxide reductase subunit c OS=Stenotrophomonas maltophilia (strain K279a) GN=ahpC PE=4 SV=1 - [B2FPY7_STRMK]	0.000	7.71
B2FPY9	Proline iminopeptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=pip PE=3 SV=1 - [B2FPY9_STRMK]	<0.05	>100

B2FQ15	Glutamyl-tRNA reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=hemA PE=3 SV=1 - [HEM1_STRMK]	<0.05	0.18
B2FQ17	Outer-membrane lipoprotein LolB OS=Stenotrophomonas maltophilia (strain K279a) GN=lolB PE=3 SV=1 - [B2FQ17_STRMK]	0.038	0.48
B2FQ28	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0885 PE=3 SV=1 - [B2FQ28_STRMK]	0.005	0.41
B2FQ31	Elongation factor Tu OS=Stenotrophomonas maltophilia (strain K279a) GN=tufB PE=3 SV=1 - [B2FQ31_STRMK]	0.001	3.65
B2FQ33	Transcription termination/antitermination protein NusG OS=Stenotrophomonas maltophilia (strain K279a) GN=nusG PE=3 SV=1 - [B2FQ33_STRMK]	0.036	2.41
B2FQ36	50S ribosomal protein L10 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplJ PE=3 SV=1 - [RL10_STRMK]	0.001	2.54
B2FQ38	DNA-directed RNA polymerase subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoB PE=3 SV=1 - [RPOB_STRMK]	0.013	5.30
B2FQ39	DNA-directed RNA polymerase subunit beta' OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoC PE=3 SV=1 - [RPOC_STRMK]	0.006	7.89
B2FQ42	Elongation factor G OS=Stenotrophomonas maltophilia (strain K279a) GN=fusA PE=3 SV=1 - [EFG_STRMK]	0.003	4.86
B2FQ57	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2204 PE=4 SV=1 - [B2FQ57_STRMK]	0.049	0.47
B2FQ62	Putative enoyl-CoA hydratase/isomerase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2209 PE=4 SV=1 - [B2FQ62_STRMK]	<0.05	0.44
B2FQ73	Transcription elongation factor GreA OS=Stenotrophomonas maltophilia (strain K279a) GN=greA PE=3 SV=1 - [B2FQ73_STRMK]	<0.05	>100
B2FQ89	Oxygen-dependent choline dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=betA PE=3 SV=1 - [BETA_STRMK]	0.007	0.35
B2FQD9	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3569 PE=4 SV=1 - [B2FQD9_STRMK]	<0.05	>100
B2FQE1	Putative transmembrane sulfatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3571 PE=4 SV=1 - [B2FQE1_STRMK]	0.004	0.22
B2FQE3	Putative angiotensin-converting enzyme like peptidyl dipeptidase protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3574 PE=4 SV=1 - [B2FQE3_STRMK]	0.018	0.37
B2FQH7	Citrate synthase OS=Stenotrophomonas maltophilia (strain K279a) GN=prpC PE=3 SV=1 - [B2FQH7_STRMK]	<0.05	0.38
B2FQJ2	Putative two-component histidine kinase/response regulator fusion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3625 PE=4 SV=1 - [B2FQJ2_STRMK]	0.040	0.41
B2FQK3	50S ribosomal protein L15 OS=Stenotrophomonas maltophilia (strain K279a) GN=rplO PE=3 SV=1 - [B2FQK3_STRMK]	0.006	2.18
B2FQK4	Protein translocase subunit SecY OS=Stenotrophomonas maltophilia (strain K279a) GN=secY PE=3 SV=1 - [B2FQK4_STRMK]	0.015	0.40
B2FQK8	DNA-directed RNA polymerase subunit alpha OS=Stenotrophomonas maltophilia (strain K279a) GN=rpoA PE=3 SV=1 - [RPOA_STRMK]	0.002	3.72
B2FQL7	Peptidyl-prolyl cis-trans isomerase OS=Stenotrophomonas maltophilia (strain K279a) GN=ppi PE=3 SV=1 - [B2FQL7_STRMK]	0.003	2.10
B2FQL8	Malate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=mdh PE=3 SV=1 - [MDH_STRMK]	0.000	3.96
B2FQM3	Putative glutathione S-transferase OS=Stenotrophomonas maltophilia (strain K279a) GN=gst PE=4 SV=1 - [B2FQM3_STRMK]	<0.05	>100
B2FQN2	Putative 2-amino-3-ketobutyrate coenzyme A ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=kbl PE=4 SV=1 - [B2FQN2_STRMK]	<0.05	2.22
B2FQN3	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0960 PE=4 SV=1 - [B2FQN3_STRMK]	0.003	0.33
B2FQN4	L-threonine 3-dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=tdh PE=3 SV=1 - [TDH_STRMK]	<0.05	>100
B2FQQ5	Putative isocitrate/isopropylmalate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0982 PE=3 SV=1 - [B2FQQ5_STRMK]	0.001	4.75
B2FQR3	ATP-dependent Clp protease ATP-binding subunit ClpX OS=Stenotrophomonas maltophilia (strain K279a) GN=clpX PE=3 SV=1 - [CLPX_STRMK]	0.025	2.39
B2FQS5	Putative flagellar basal body-associated protein FliL OS=Stenotrophomonas maltophilia (strain K279a) GN=fliL PE=4 SV=1 - [B2FQS5_STRMK]	<0.05	0.25
B2FQU5	Putative flagellin OS=Stenotrophomonas maltophilia (strain K279a) GN=fliC PE=4 SV=1 - [B2FQU5_STRMK]	0.038	2.62
B2FQU6	Putative flagellin OS=Stenotrophomonas maltophilia (strain K279a) GN=flaA PE=4 SV=1 - [B2FQU6_STRMK]	0.034	2.68
B2FQY3	Thioredoxin reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=trxB PE=3 SV=1 - [B2FQY3_STRMK]	<0.05	>100
B2FR07	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3644 PE=4 SV=1 - [B2FR07_STRMK]	<0.05	>100
B2FR21	Aminomethyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=gcvT PE=3 SV=1 - [GCST_STRMK]	<0.05	0.27

B2FR42	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3680 PE=4 SV=1 - [B2FR42_STRMK]	0.024	0.42
B2FR45	Putative O-antigen related protein OS=Stenotrophomonas maltophilia (strain K279a) GN=wbpV PE=4 SV=1 - [B2FR45_STRMK]	0.017	0.46
B2FR62	Putative autotransporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1001 PE=4 SV=1 - [B2FR62_STRMK]	<0.05	0.04
B2FR74	Putative DNA polymerase III subunit Tau OS=Stenotrophomonas maltophilia (strain K279a) GN=dnaX PE=4 SV=1 - [B2FR74_STRMK]	<0.05	0.48
B2FR82	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1022 PE=4 SV=1 - [B2FR82_STRMK]	0.001	0.20
B2FR90	Acyl carrier protein OS=Stenotrophomonas maltophilia (strain K279a) GN=acpP PE=3 SV=1 - [B2FR90_STRMK]	<0.05	>100
B2FR91	3-oxoacyl-[acyl-carrier-protein] synthase 2 OS=Stenotrophomonas maltophilia (strain K279a) GN=fabF PE=3 SV=1 - [B2FR91_STRMK]	<0.05	>100
B2FRC4	Putative TonB-dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1067 PE=3 SV=1 - [B2FRC4_STRMK]	0.024	3.11
B2FRE3	Putative esterase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2353 PE=4 SV=1 - [B2FRE3_STRMK]	<0.05	>100
B2FRE4	Putative ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2354 PE=4 SV=1 - [B2FRE4_STRMK]	<0.05	>100
B2FRE5	Putative binding-protein-dependent transport lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2355 PE=4 SV=1 - [B2FRE5_STRMK]	0.000	19.84
B2FRE6	Putative FecCD-family transmembrane transport protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2356 PE=4 SV=1 - [B2FRE6_STRMK]	<0.05	>100
B2FRE7	Hemin import ATP-binding protein HmuV OS=Stenotrophomonas maltophilia (strain K279a) GN=hmuV PE=3 SV=1 - [B2FRE7_STRMK]	<0.05	>100
B2FRM8	Putative peptidoglycan-associated lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3703 PE=3 SV=1 - [B2FRM8_STRMK]	0.005	0.42
B2FRN1	Putative TolR-related protein OS=Stenotrophomonas maltophilia (strain K279a) GN=tolR PE=3 SV=1 - [B2FRN1_STRMK]	0.013	0.42
B2FRN2	Putative TolQ transport transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=tolQ PE=3 SV=1 - [B2FRN2_STRMK]	0.018	0.35
B2FRR0	Putative lipoprotein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3739 PE=4 SV=1 - [B2FRR0_STRMK]	0.001	0.39
B2FRU0	Pyrroline-5-carboxylate reductase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1087 PE=3 SV=1 - [B2FRU0_STRMK]	<0.05	>100
B2FRV6	Putative MgtE/divalent cation transmembrane transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1103 PE=4 SV=1 - [B2FRV6_STRMK]	0.028	0.47
B2FRW5	Putative ABC transporter, ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1113 PE=4 SV=1 - [B2FRW5_STRMK]	0.014	0.50
B2FRX9	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1127 PE=4 SV=1 - [B2FRX9_STRMK]	0.013	0.49
B2FRZ2	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1140 PE=4 SV=1 - [B2FRZ2_STRMK]	<0.05	14.83
B2FRZ3	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1141 PE=4 SV=1 - [B2FRZ3_STRMK]	<0.05	>100
B2FRZ9	Putative iron transport receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1148 PE=3 SV=1 - [B2FRZ9_STRMK]	0.000	2.21
B2FS06	Putative ABC transporter, ATP-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1155 PE=4 SV=1 - [B2FS06_STRMK]	<0.05	2.41
B2FS15	Putative histone-like protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1164 PE=4 SV=1 - [B2FS15_STRMK]	0.000	4.96
B2FS22	Putative copper-transporting p-type ATPase OS=Stenotrophomonas maltophilia (strain K279a) GN=copF PE=3 SV=1 - [B2FS22_STRMK]	<0.05	0.17
B2FSD2	Putative transmembrane transporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3777 PE=4 SV=1 - [B2FSD2_STRMK]	0.014	0.41
B2FSE0	Putative transmembrane symporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3785 PE=4 SV=1 - [B2FSE0_STRMK]	0.017	0.36
B2FSE3	Putative HlyD-family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeM PE=4 SV=1 - [B2FSE3_STRMK]	0.001	0.37
B2FSE4	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3789 PE=3 SV=1 - [B2FSE4_STRMK]	0.006	2.18
B2FSE9	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3796 PE=4 SV=1 - [B2FSE9_STRMK]	0.001	0.28
B2FSF0	Fructose-bisphosphate aldolase OS=Stenotrophomonas maltophilia (strain K279a) GN=alf1 PE=3 SV=1 - [B2FSF0_STRMK]	0.000	2.71
B2FSF3	Phosphoglycerate kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=pgk PE=3 SV=1 - [PGK_STRMK]	<0.05	3.06
B2FSF6	Glyceraldehyde-3-phosphate dehydrogenase OS=Stenotrophomonas maltophilia (strain K279a) GN=gap PE=3 SV=1 - [B2FSF6_STRMK]	0.001	3.04

B2FSF7	Putative outer membrane Omp family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3805 PE=4 SV=1 - [B2FSF7_STRMK]	0.045	0.10
B2FSF8	Putative endonuclease P1 OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3806 PE=4 SV=1 - [B2FSF8_STRMK]	0.004	0.45
B2FSG4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3812 PE=4 SV=1 - [B2FSG4_STRMK]	0.016	0.38
B2FSG6	Putative transmembrane sodium-dicarboxylate family transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3814 PE=4 SV=1 - [B2FSG6_STRMK]	0.025	0.42
B2FSH0	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3818 PE=4 SV=1 - [B2FSH0_STRMK]	<0.05	0.46
B2FSH4	Putative pilP protein (Type 4 fimbrial biogenesis protein) OS=Stenotrophomonas maltophilia (strain K279a) GN=pilP PE=4 SV=1 - [B2FSH4_STRMK]	<0.05	0.32
B2FSH6	Putative PilN protein (Type 4 fimbrial biogenesis protein) OS=Stenotrophomonas maltophilia (strain K279a) GN=pilN PE=4 SV=1 - [B2FSH6_STRMK]	<0.05	0.35
B2FSH7	Putative PilM protein (Type 4 fimbrial biogenesis protein) OS=Stenotrophomonas maltophilia (strain K279a) GN=pilM PE=4 SV=1 - [B2FSH7_STRMK]	0.038	0.21
B2FSJ9	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1175 PE=4 SV=1 - [B2FSJ9_STRMK]	<0.05	>100
B2FSN4	Putative transmembrane phosphoesterase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1210 PE=4 SV=1 - [B2FSN4_STRMK]	0.017	0.31
B2FSQ7	Putative TonB-dependent receptor for Fe(III)-coprogen, Fe(III)-ferrioxamine B and Fe(III)-rhodotrulic acid OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1233 PE=3 SV=1 - [B2FSQ7_STRMK]	0.000	41.01
B2FT31	Putative ACR family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3869 PE=4 SV=1 - [B2FT31_STRMK]	0.014	0.44
B2FT45	Putative transmembrane DedA family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3883 PE=4 SV=1 - [B2FT45_STRMK]	0.030	0.50
B2FT59	Putative extracellular heme-binding protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3898 PE=3 SV=1 - [B2FT59_STRMK]	0.000	8.18
B2FT63	Inorganic pyrophosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=ppa PE=3 SV=1 - [B2FT63_STRMK]	<0.05	2.30
B2FT66	Putative TonB dependent receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3905 PE=3 SV=1 - [B2FT66_STRMK]	0.014	0.44
B2FT80	DNA polymerase III subunit beta OS=Stenotrophomonas maltophilia (strain K279a) GN=dnaN PE=4 SV=1 - [B2FT80_STRMK]	<0.05	>100
B2FT86	Conserved hypothetical TPR repeat family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0008 PE=4 SV=1 - [B2FT86_STRMK]	0.000	4.78
B2FT87	**Putative proline-rich TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0009 PE=4 SV=1 - [B2FT87_STRMK]**	**0.006**	**0.34**
B2FTA3	Putative exported peptidase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1246 PE=4 SV=1 - [B2FTA3_STRMK]	0.033	2.06
B2FTA8	Probable septum site-determining protein MinC OS=Stenotrophomonas maltophilia (strain K279a) GN=minC PE=3 SV=1 - [B2FTA8_STRMK]	<0.05	2.27
B2FTD0	UvrABC system protein A OS=Stenotrophomonas maltophilia (strain K279a) GN=uvrA PE=3 SV=1 - [B2FTD0_STRMK]	<0.05	0.45
B2FTJ0	Isoleucine--tRNA ligase OS=Stenotrophomonas maltophilia (strain K279a) GN=ileS PE=3 SV=1 - [SYI_STRMK]	<0.05	2.17
B2FTK5	Putative ferric siderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2650 PE=3 SV=1 - [B2FTK5_STRMK]	<0.05	5.09
B2FTN0	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2678 PE=4 SV=1 - [B2FTN0_STRMK]	<0.05	0.32
B2FTR3	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2712 PE=4 SV=1 - [B2FTR3_STRMK]	<0.05	>100
B2FTR4	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2713 PE=4 SV=1 - [B2FTR4_STRMK]	<0.05	>100
B2FTR5	Putative TonB dependent protein, possible siderophore receptor OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt2714 PE=3 SV=1 - [B2FTR5_STRMK]	<0.05	>100
B2FTS4	Uncharacterized protein OS=Stenotrophomonas maltophilia (strain K279a) GN=pcm PE=4 SV=1 - [B2FTS4_STRMK]	0.007	0.38
B2FTS5	Protein CyaE OS=Stenotrophomonas maltophilia (strain K279a) GN=tolC PE=3 SV=1 - [B2FTS5_STRMK]	0.001	0.25
B2FTS8	Putative lipid A biosynthesis lauroyl acyltransferase OS=Stenotrophomonas maltophilia (strain K279a) GN=htrB PE=4 SV=1 - [B2FTS8_STRMK]	0.017	0.44
B2FTT7	Putative porin P (Outer membrane protein d1) OS=Stenotrophomonas maltophilia (strain K279a) GN=oprP PE=4 SV=1 - [B2FTT7_STRMK]	0.007	0.45
B2FTV4	Putative glutaredoxin OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3961 PE=4 SV=1 - [B2FTV4_STRMK]	<0.05	2.73

B2FTV7	Conserved hypothetical exported protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3964 PE=4 SV=1 - [B2FTV7_STRMK]	0.024	0.38
B2FTY9	Putative endonuclease/exonuclease/phosphatase family protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt3995 PE=4 SV=1 - [B2FTY9_STRMK]	<0.05	0.36
B2FU12	Putative iron transporter protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0049 PE=3 SV=1 - [B2FU12_STRMK]	0.049	5.51
B2FU40	Putative patatin-like phospholipase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0080 PE=4 SV=1 - [B2FU40_STRMK]	0.005	0.42
B2FU42	Putative TonB dependent receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0083 PE=3 SV=1 - [B2FU42_STRMK]	0.001	0.24
B2FU43	Acid phosphatase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt0084 PE=3 SV=1 - [B2FU43_STRMK]	0.000	0.20
B2FU51	Thymidine kinase OS=Stenotrophomonas maltophilia (strain K279a) GN=tdk PE=3 SV=1 - [B2FU51_STRMK]	<0.05	>100
B2FU87	Putative transmembrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1346 PE=4 SV=1 - [B2FU87_STRMK]	0.012	0.32
B2FU91	Putative outer membrane autotransporter OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1350 PE=4 SV=1 - [B2FU91_STRMK]	<0.05	>100
B2FUA1	Putative quinol oxidase subunit 1 OS=Stenotrophomonas maltophilia (strain K279a) GN=qoxB PE=3 SV=1 - [B2FUA1_STRMK]	0.011	0.32
B2FUA6	Putative chaperone protein HtpG (Heat shock protein HtpG) OS=Stenotrophomonas maltophilia (strain K279a) GN=htpG PE=4 SV=1 - [B2FUA6_STRMK]	<0.05	0.36
B2FUD9	Putative HlyD family secretion protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1406 PE=4 SV=1 - [B2FUD9_STRMK]	0.011	0.43
B2FUF0	Putative nucleotide sugar transaminase OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt1417 PE=3 SV=1 - [B2FUF0_STRMK]	<0.05	>100
B2FUL1	Putative respiratory nitrate reductase subunit OS=Stenotrophomonas maltophilia (strain K279a) GN=narH PE=4 SV=1 - [B2FUL1_STRMK]	0.018	0.28
B2FUN9	Putative TonB-dependent receptor for Fe(III)-coprogen, Fe(III)-ferrioxamine B and Fe(III)-rhodotrulic acid OS=Stenotrophomonas maltophilia (strain K279a) GN=fhuE PE=3 SV=1 - [B2FUN9_STRMK]	0.000	13.58
B2FUR1	Putative TonB-dependent outer membrane receptor protein OS=Stenotrophomonas maltophilia (strain K279a) GN=Smlt4026 PE=3 SV=1 - [B2FUR1_STRMK]	0.001	0.13
B2FUT3	Putative exported rare lipoprotein A OS=Stenotrophomonas maltophilia (strain K279a) GN=rlpA PE=3 SV=1 - [B2FUT3_STRMK]	0.001	0.27
B2FUT4	Putative murein hydrolase OS=Stenotrophomonas maltophilia (strain K279a) GN=mltB PE=4 SV=1 - [B2FUT4_STRMK]	0.011	0.46
B2FUT9	Putative rod shape-determining protein OS=Stenotrophomonas maltophilia (strain K279a) GN=mreC PE=4 SV=1 - [B2FUT9_STRMK]	0.009	0.44
B2FUV1	Putative multidrug resistance outer membrane protein OS=Stenotrophomonas maltophilia (strain K279a) GN=smeF PE=4 SV=1 - [B2FUV1_STRMK]	0.002	0.39
B2FUV2	Putative acriflavin resistance protein B OS=Stenotrophomonas maltophilia (strain K279a) GN=smeE PE=4 SV=1 - [B2FUV2_STRMK]	0.013	0.31
B2FUV3	Putative acriflavin resistance protein A OS=Stenotrophomonas maltophilia (strain K279a) GN=smeD PE=4 SV=1 - [B2FUV3_STRMK]	0.002	0.32

7 General discussion

A major aim of this work was to test the efficacy of last-line and experimental antimicrobials by taking advantage of the better understanding of the mechanisms of resistance in *S. maltophilia* obtained through parallel work characterising novel resistance mechanisms. By performing this, we have managed to highlight the importance of surveying this particular bacterium for it is an extremely interesting model to study antimicrobial resistance given the presence of multiple intrinsic mechanisms but foremost for its increasing prevalence during the last few decades in severely debilitated and cystic fibrosis patients.

The initial aim of the project was to characterise mechanism of resistance against some antimicrobial alternatives to co-trimoxazole for treating *S. maltophilia* infections that include: ceftazidime, amikacin, moxifloxacin, levofloxacin, and minocyline. In **Chapter 3**, the aim was to characterise ceftazidime resistant mutants. Whole genome sequencing allowed to find a novel cause of β-lactamase hyperproduction. The *mpl* gene that encodes a murein protein ligase also known as UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase was found to have a loss of function mutation that affects the joining of the polypeptide (356) released during cell wall recycling to UDP-N-acetylmuramate. When we assessed the role of *mpl* in β-lactamase activity and consequently in ceftazidime resistance, *trans*-complementation with the wild-type version of *mpl* returned β-lactamase activity to basal levels and restored susceptibility to ceftazidime. Therefore, we hypothesised that *mpl* loss will trigger accumulation of the penta-peptides which ultimately inhibit activity of the amidase, AmpD, despite the degradation of those penta-peptides by peptidases such as LdcA, MpaA, YcjI and PepD (222). Losing AmpD activity will affect the release of penta-peptide from the muropeptide and generate an increased presence of the anhydro-muramyl-penta-peptide which act as an activator ligand for the transcriptional regulator of L2 and L1. Interestingly, *mpl* mutations were not only common in laboratory selected β-lactamase hyperproducing mutants (30% of the cases) but more important in clinical isolates (75%). In *P. aeruginosa* isolates recovered from cystic fibrosis patients and patients with ventilator associated pneumonia, *mpl* loss has been observed (238) but the significance of this finding has been underestimated. In our small collection of *P. aeruginosa* clinical isolates 40% carried an *mpl* mutation. Therefore, we concluded that *mpl* loss is an important cause for β-lactamase production not only in *S. maltophilia* but

P. aeruginosa, and that loss might arise during a long-term β-lactam therapy in infected patients.

Following the aim of **Chapter 3,** we then moved on to the characterisation of non-β-lactamase hyperproducers. This time it was the combination of whole genome sequencing and comparative proteomics the strategy that helped us to propose a totally novel mechanism of ceftazidime resistance. Initial antimicrobial characterisation showed pan β-lactam resistance despite exhibiting basal level of β-lactamase activity. When using dye accumulation assays to test envelope permeability, we found reduction. It is important to mention that the cellular membrane of *S. maltophilia* is inherently less permeable when compared to other bacteria due to its reduced number of porins (245), so that might explain why there are no reports of loss of function porin mutations that explain reduced antibiotic uptake and why reduction in permeability observed in non-β-lactamase producers might be explained by a different reason other than porin reduction. Analysis of whole genome sequencing showed mutations within the proline-rich TonB energy transducer Smlt0009. Knock out of the gene caused ceftazidime resistance. Therefore, we hypothesize that losing the proline rich TonB energy transducer impedes energy transduction, generated through proton motive force, to enable the conformational shift of the ligand gated porin for the uptake of a broad range of substrates, including iron/siderophores and highly likely β-lactams or at least ceftazidime. In fact, when testing envelope permeability to a fluorescent dye in presence of ceftazidime, permeability decreased when the proline rich TonB energy transducer was intact – suggesting competition for import - but permeability remained the same when the proline rich TonB energy transducer was altered – suggesting lack of ceftazidime import, and so lack of competition with the dye. Proteomics confirmed downregulation of the proline rich TonB energy transducer but upregulation of proteins involved in siderophore synthesis. When testing phenotypically, we did observe more siderophore production in the non-β-lactamase hyperproducers which is likely to be in response to low iron uptake, a fundamental element for all forms of life (357). However, in terms of virulence, loss of the proline rich TonB energy transducer might not be significant advantageous. In order to test this it would be interesting to analyse the sequence of the proline rich TonB energy transducer of the many ceftazidime resistant non-β-lactamase hyperproducing clinical isolates identified in our worldwide collection.

In **Chapter 4** we aimed to gain a better understanding about the mechanisms of resistance involved in aminoglycoside, quinolone and tetracycline resistance. Whole envelope proteomics confirmed upregulation of *smeYZ* in the amikacin resistant profile which also includes reduction in susceptibility to quinolones and tetracyclines

(resistance profile 3). However, when analysing genome sequencing we found mutations in the *rplA* gene which encodes the ribosomal unit, L1. In *P. aeruginosa*, mutations in other ribosomal subunit genes have been found to induce expression of *mexXY*, the most common RND-efflux pump involved in aminoglycoside resistance in *P. aeruginosa* (358). In order to test if ribosomal damage was responsible of enhanced expression of *smeYZ*, we measured expression of *smeYZ* after induction with a ribosomal-acting antibiotic, the aminoglycoside gentamicin, and confirmed hyperexpression of *smeYZ*. Clinical implications of this finding is an increased virulence, given the physiological role of SmeYZ in motility, protease secretion, and biofilm formation. In fact, laboratory selected mutants exhibited increased swimming motility and an *rplA* mutation was found in a clinical isolate with resistance profile 3. Continuing with the physiological role of *smeYZ,* it has been suggested that its downregulation might be compensated by overexpression of *smeDEF* (180). In fact, this was a particular characteristic of resistant profile 1 mutants (resistant to quinolones and tetracyclines but susceptible to aminoglycosides) that according to proteomics SmeYZ was downregulated as SmeDEF was being upregulated. One of the reasons for SmeDEF hyperproduction was an activator mutation Thr84Pro in the local repressor SmeT, previously reported. Alternatively, a mutation found the glycosyltransferase (Gly368Ala) encoded by *smlt0622*, possibly involved in lipid synthesis, might trigger a different signal for SmeDEF constitutive production. These mutations might imply a different signal in the interplay between SmeSyRy, local regulator of SmeYZ, and SmeT, local regulator of SmeDEF to try to preserve or improve the infection potential.

Continuing with quinolone resistance mechanisms, we then characterised a levofloxacin resistant mutant which also belonged to the previously mentioned resistance profile 1, generated as a derivative of a SmeDEF hyperproducer with a mutated glycosyltransferase. In the whole genome, we confirmed the mutation in the glycosyltransferase but also found a mutation in the Smlt2645-6 two-component system which is the first candidate to regulate the expression of an ABC transporter annotated as MacAB that according to proteomics results was upregulated. Complementation *in trans* with the mutated version of the operon containing the two-component system proved to be sufficient to confer resistance to levofloxacin regardless the presence of a mutated glycosyltransferase. However, the mutation in the glycosyltransferase might ease the acquisition of the mutation in the two-component system since it was only possible to generate second-step resistant mutants.

Despite our attempts to generate minocycline resistant mutants we could only obtain a second-step mutant with reduced susceptibility that fitted in the resistance profile 1. Two mutations were identified in the whole genome, one in a lauroyl acyltransferase and one in the Smlt1635-6 two-component system. The two component system is located next to a 10-component operon where the putative Smlt1640 ABC transporter is located and that according to proteomics is upregulated. An ABC transporter homologue in *E. coli* has two binding sites, whilst one participates in lipid trafficking, the other is involved in drug transport (278). Other ABC transporters involved in lipid transport have been found to be associated with minocycline resistance. Upregulation of the *mla*FEDCB operon which encodes the ABC transporter would be involved in decreased permeability to hydrophobic substrates such as minocycline (280) by a reduction of phospholipids in the outer leaftet of the outer membrane. According to proteomics there was also SmeDEF upregulation which has been found to be involved in tetracycline resistance (168). We could not find a common explanation for *smeDEF* upregulation. However, if we combine the mutation found in lauroyl acyltransferase and the glycosyltransferase, above mentioned, it seems possible to suggest that alterations in lipid synthesis might be a signal to induce *smeDEF* upregulation.

Chapter 5 was built on the previous chapters where effectiveness of β-lactamase inhibitors was assessed once we had a better understanding of the mechanism of resistance present in *S. maltophilia.* Establishing hydrolysis efficiency of both β-lactamases produced in *S. maltophilia*, L1 and L2, against some β-lactams led us to prove that ceftazidime showed to be only poorly hydrolysed by both enzymes, whilst meropenem was more efficiently hydrolysed by L1 and aztreonam by L2. In the cell, the poor hydrolytic activity either by low enzymatic affinity or slow enzymatic hydrolysis, is mainly compensated by β-lactamase hyperproduction (359) as shown in **Chapter 3.** Hence the need of efficient β-lactamase inhibitors. When testing the ability of clavulanic acid, avibactam and a recently designed bicyclic boronate to rescue β-lactam activity from β-lactamase production; we observed that none of the inhibitors restored meropenem activity but all reversed aztreonam and ceftazidime resistance. In addition, its effectiveness is not reduced when cell permeability is altered by overproduction of the efflux-pumps SmeYZ and SmeDEF as described in **Chapter 4.** All observations combined concluded that inhibitors are effective but their activity is mainly exerted on the SBL, L2, but not on the MBL subclass 3, L1, predominantly based on the unrestored meropenem activity even the bicyclic boronate that had demonstrated to inhibit other MBLs (293). In fact, β-lactamase inhibitors were

kinetically active against L2 with nanomolar potencies ranging from 5.25 to 22.3 nM, where bicyclic boronate exhibited the lowest; but not L1 even at milimolar concentrations of the inhibitor. Clavulanic acid was excluded for further analysis since it showed to induce L1 hyperproduction when compared to avibactam and bicyclic boronate **2**. Analysing the structural basis for inhibition of L2 by the bicyclic boronate **2** corroborated the covalent binding of both inhibitors in the active site. Bicyclic boronate **2**, showed a similar conformation when compared to the CTX-M-15:bicyclic boronate **1** (310) complex including the tetrahedral boron atom and the fused bicyclic core with a slight variation in the amide/benzamide side chain. Avibactam, also showed a similar conformation when compared to other class A SBLs (CTX-M-15 (332), KPC-2 (331), SHV-1(331)) where avibactam is in an opened chair conformation to establish the carbamoyl-L2 complex with some differences establishing the importance of the Asn132 and Thr235 for interaction with avibactam in L2. Despite microbiological, kinetic, and structural confirmation of inhibition of L2 by avibactam and bicyclic boronate we predicted failure for the last-line combination ceftazidime/avibactam and ceftazidime/ bicyclic boronate since selected mutants will overcome their effectivity by L1 hyperproduction. However, we proposed aztreonam/avibactam and aztreonam/bicyclic boronate as alternatives to treat infections caused by β-lactamase hyperproduction. In the search of providing more choices to a possibly emerging L1 production, other experimental MBL-inhibitors were tested. A phosphonate based inhibitor, 6-phosphonomethylpyridine-2-carboxylate, showed to be a promising scaffolding structure since its activity in the cell was not affected by overproduction of efflux pumps, at least SmeYZ; however the high concentrations required to inhibit L1, limit its use. Interestingly, recently designed rhodanine based inhibitors have shown nanomolar potencies (316) and here restored meropenem activity in the mutants described in **Chapter 3** and **4** but not in those generated in **Chapter 5** where aztreonam/avibactam and aztreonam/ bicyclic boronate **2** combinations were still effective**.** This highlights the importance of considering the administration of aztreonam in combination with a SBL inhibitor in *S. maltophilia* when β-lactamase hyperproduction is suspected as it has occurred in the treatment of infections caused by Enterobacteriaceae (340).

In **Chapter 6** we aimed to evaluate the antimicrobial activity of lactivicin derivatives against the mutants generated in **Chapter 3**, **4** and extensively drug resistant clinical isolates; and characterise possible emerging lactivicin resistance mechanisms. The antimicrobial potency of the lactivicin derivative carrying a catechol-type sideromimic modification showed to be particularly high in *S. maltophilia* including

a collection of extensively drug resistant clinical isolates and the mutants described in **Chapter 3** and **4.** Even though, the lactivicin derivative showed to be hydrolysed by L1 but not L2, L1 activity is too slow to abolish the antimicrobial activity of the LTV derivative. Recently it has been shown that *S. maltophilia* possesses the machinery to synthesize catechol-type siderophores (195, 249) which might explain the increased uptake of catechol based antibiotics. However, it was interesting to find reduced LTV susceptibility in the mutants with loss of the TonB energy transducer described in **Chapter 3**. In fact, when selecting mutants with reduced susceptibility in presence of the LTV carrying the sideromimic modification, loss of the proline-rich region in the TonB energy transducer was found again. Additionally, whole envelope proteomics showed, downregulation of TonB energy transducer and increased siderophore production. This would add further evidence to the hypothesis stated in **Chapter 3** where loss of TonB energy transducer limits the uptake of myriad substances including ceftazidime and catechol-type siderophores involved in iron trafficking, and therefore catechol-based antimicrobials. In fact, reports suggest that diminished uptake of siderophore conjugated drugs is due to the presence of mutations in the LGP or the ferric uptake regulator (Fur) (351, 353). However, since there is no standardized breakpoint for lactivicin derivatives, it would be possible to consider that although its activity is diminished, still preserves good antimicrobial activity. Nevertheless, this finding is worth of consideration when designing siderophore based drugs.

Future work for additional confirmation of the role on TonB energy transducer in ceftazidime resistance and lactivicin reduced susceptibility will include a survey of the sequence of *smlt0009* in the many ceftazidime resistant clinical isolates identified that do not hyper-produce β-lactamase . In the case of levofloxacin resistant mutants and moxifloxacin and minocycline mutants with reduced susceptibility, it remains to be tested if the mutations found in the glycosyltransferase (*encoded by smlt0622*) and lauroyl acyltransferase (*smlt4167*) are enough to reduce susceptibility to levofloxacin and minocycline, respectively or if they have an interplay with the transcriptional regulator SmeT to trigger the upregulation of *smeDEF*. Testing this will involve the knock-out of both genes separately in a wild-type background or alternatively knocking-out *smeE* in the mutants. For SmeYZ hyperproducers with reduced aminoglycoside susceptibility, it remains to be testes if the *rplA* mutation seen is enough to trigger reduction of aminoglycoside susceptibility or if the phenotype definitely requires the overproduction of the SmeYZ efflux-pump. This can be tested with complementation *in trans* with the wild type *rplA* gene or the mutated version in a background lacking the pump where susceptibility would remain the same or only slightly different if the *rplA*

mutation was not sufficient to cause resistance on its own. To perform this it would be preferable the use of a vector with a non-ribosomal-acting selection marker which could be a tellurite cassette. Despite applying different methods for analysing the genome sequence of the mutants generated in **Chapter 5** we could not find an explanation for L1 hyperproduction, thus it would be preferable to re-sequence the genomes of the mutants and parent strain using long-read technology such as MinION. Since the search of a pan β-lactamase inhibitor will continue, it would be important to attempt generation and characterisation of mutants against the MBL inhibitors here mentioned to contribute to a rational design and propose new alternatives for treatment of *S. maltophilia* infections. It would be also interesting to find analogues of catechol conjugated lactivicins or other drugs.

In conclusion, a lot of important and clinically relevant information has been identified about *S. maltophilia* in this study, and what we have learned will be particularly important for the progression of novel and/or current last resort agents against this species.

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